TAXONOMY AND GENERAL ECOLOGY OF MARINE INVERTEBRATES FROM LAS PERLAS ARCHIPELAGO, PANAMANIAN TROPICAL EASTERN PACIFIC

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ABSTRACT

This project focused on two main marine invertebrate groups: firstly, a series of reefs composed of the Sabellarid polychaete Idanthyrsus cretus Chamberlain, 1919 (not previously described as forming reefs) was found in the south-eastern part of Las Perlas Archipelago (Pacific of Panama) in areas of high exposure. The aim was to reveal its potential distribution patterns in relation to physical data and geographical origins, including a molecular approach. Analysis showed that their reef distribution in the archipelago appeared to be mainly dependent on depth, suitable substrate and topography, available sediment particle size for tube building and wave exposure. Studies indicated that I. cretus reefs may be offering physical and ecological space to a large diversity of associated fauna that in other parts of the archipelago are provided by the coral reefs. The polychaete reefs in the area corresponded to one population that probably migrated from the south, from Colombia or Ecuador. The second marine invertebrate studied was the vermetid (Mollusca) Eualetes tulipus (Chenu 1843, ex Rouseau). Here the aim was to describe its taxonomy and ecology, and to demonstrate its potential as a proxy indicator of changes in SST and Salinity for the TEP. A new morphotype of the species was described, with a particularly long tube-shaped shell, associated with Pocillopora coral reef environments in Las Perlas. Microhabitat requirements of the new morphotype were for areas with high cover (>80%) and high density of branched corals and a minimal vertical growth of coral (>40 cm). Analyses from Panama shells showed a growth rate of 24mm/year and detectable annual, monthly and possibly daily bands. Results of X-ray diffraction and SEM analysis showed aragonite as the main shell component with oxygen isotopes not in isotopic equilibrium with the sea water but nearer to the isotopic equilibrium than corals. A positive relationship between the Mg and SST and a negative relationship between the Sr and SST was demonstrated and also a negative relationship between δ18O and SST. The relationship between the trace elements and oxygen isotopes with the records for SST and salinity are in general agreement with previous literature for other marine invertebrates. Comparisons between methodologies used for the analysis of trace elements showed ICP-OES as the best methodology in terms of accuracy and cost. Comparisons between methodologies used for oxygen isotopes analysis showed that MS was good for general growth band pattern analysis. However, when more details and/or minimal amount of sample are needed, IMF is considered the best option.

DEDICATION

To my men,

the light of my life,

my loved Takis and my little Santiago.

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LIST OF PUBLICATIONS IN PREPARATION BY THE CANDIDATE

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Barrios L.M., Elliot M., Mair J.M. 2007. Vermetids as archives of environmental change in the Pacific coast of Panama.

Barrios L.M., Chambers S., Ismail N., Mair J.M. 2007. Distribution of *Idanthyrsus cretus* (Sabellaridae:Polychaeta) in the Tropical Eastern Pacific-TEP and application of PCR-RAPD's tools for population analysis.

Chapter 1. General introduction

This work is part of a common Darwin Initiative research project, a collaborative effort between Heriot Watt University (UK) and the Smithsonian Tropical Research Institute-STRI (Panama). The main objective of the Darwin project was to identify the main fauna and flora of the Las Perlas Archipelago (Pacific of Panama) in order to establish a base line study for the area, the key ecological aspects and the main threats to the area which is being proposed as a protected area by the national authorities.

Las Perlas Archipelago (8º00'-8º40'N/78º30'-79º12'W; Figure 1) is part of the Tropical Eastern Pacific bio-geographic zone which extends from 23º26'N in the Gulf of California, Mexico to 1º28'S, southern Ecuador (Macintyre *et al.* 1992, Cortés 1997, Glynn and Mate 1997). The coral reefs in this region are typically small fringing reefs, shallow (<10 m deep), and species poor (< 24 species of coral) in comparison with the western pacific reefs that exhibit more than 500 coral species (Glynn and Mate 1997, Macintyre *et al.* 1992, Diaz *et al.* 2000, Glynn 2001a). The area is characterized by extreme physical and oceanographic conditions, especially large changes in temperatures, salinity and change in the concentration of nutrients and sedimentation due respectively to El Niño-La Niña events, upwelling and riverine discharges during the warm-wet season (Macintyre *et al.* 1992, Cortés 1997, D´Croz and Robertson 1997). The area also presents extreme low tidal exposures and intensive biological interactions (Colgan 1989, Cortés 1997, Díaz *et al.* 2000). From the geological point of view, the emergence of the Isthmus of Panama some 3.2 to 3.5 million years ago and the disruption of the seaway brought marked changes in the Middle American marine environments, affecting circulation, depth, temperature, sedimentation, nutrients and productivity (Colgan 1989, Cortés 1997, Glynn and Mate 1997, D'Croz and Robertson 1997) that are evident nowadays (e.g. ENSO). Despite all these ecological restrictions, the Las Perlas Archipelago has a surprisingly flourishing reef environment, which makes it a very interesting area and a small natural marine laboratory for ecological research.

Figure 1. Las Perlas Archipelago in the Tropical Eastern Pacific of Panama. Source http://www.reef.org/data/database.htm.

The present work was focused on two main marine invertebrate groups. In the case of the marine polychaete worms of the family Sabellaridae, a series of reefs was identified during surveys in the south-eastern part of the archipelago composed basically of the Sabellarid *Idanthyrsus cretus.* The colonies were found in areas of high exposure and since there are no previous descriptions of this type of reef formation for the species in the Tropical Eastern Pacific-TEP (only as part of coral or rocky areas) the aim of the work was to reveal potential distribution patterns for *Idanthyrsus cretus* in relation to physical data. The reasons for their distribution patterns, including a molecular approach to the analysis of populations (PCR-RAPD's analysis) and their geographical origins (chapter 10) were also studied. In this case the hypothesis was that the stations (12 stations: 4 replicates per station for molecular analysis) were composed of separate populations or from different settlement events.

The second marine invertebrate used during this work was the mollusc *Eualetes tulipus* (Family Vermetidae), which is found in several areas within the Tropical Eastern Pacific-TEP, including Panama, associated with widespread reefs of branching corals (*Pocillopora*) and rocky areas. The work in this case was focused on two main objectives. The first objective (chapter 8) was to describe the general taxonomy (including confirmation of the new morphotype associated with coral reef environments, with the last whorl partly free and erected and described as *Vermetus panamensis* var. *bridgesii* in Mörch 1861) and ecological aspects related to microhabitat and structure of associated environment (reef complexity, current reef structure, and the vermetids' distribution pattern). The second objective (chapter 9) aimed to demonstrate

the potential use of this vemetid as a proxy indicator of changes in Sea Surface Temperature-SST and Salinity for the TEP, bearing in mind that the structures of their shells have similar properties to those of the dominant coral species in the area, the branched *Pocillopora* spp, but with some extra advantages (not branched and therefore not fragmented information, less taxonomic and systematic difficulties, and the potential option that they precipitate their shells in isotopic equilibrium with sea water as do other molluscs).

It is expected that the results from this research will complement work being undertaken by several other UK and international institutions on similar marine communities. In the specific case of the vermetids, the use of this new proxy indicator could be used as an alternative species for temporal/ecological analysis of environmental change in areas where there are fewer, more scattered and threatened massive corals (e.g. the TEP). The work will also enhance the co-operation between several UK and overseas institutions (STRI, Panama) and it is intended that the results obtained from the project will be published in the scientific literature to add to the sparse but growing knowledge of specific environmental impacts on Tropical Eastern Pacific (TEP) marine environments. The research will produce valuable collated datasets of environmental variables from a major marine geographic region and relate this information to changes in structure of sabellarid reefs and vermetids associated with coral reef environments.

The first part of the document includes only one chapter, chapter 2, and contains the theoretical background that any reader would need to understand the results obtained during the work. Thus, chapter 2.1 includes a general overview of the TEP, their coral reef environments, their biological and economical importance, and the justification of the work. Section 2.2 corresponds to the description of the specific study area, the Las Perlas Archipelago in the Panamanian Pacific. Explanation of the techniques (chronology of calcium-carbonate marine organisms and Time series analysis) and variables studied for the analysis of vermetids (oxygen isotopes, the trace elements Mg, Sr, and Ba, and Sea Surface Temperature-SST), and the relationship of the studied variables and global warming is discussed in section 2.3. Section 2.4 explores the theory around the molecular tools used for the ecological analysis of the sabellarid worm population found forming reefs in the south eastern part of the Las Perlas Archipelago. The last section (2.5) in chapter 2 includes a general description of biological and

taxonomic aspects of each studied group, the mollusc *Eualetes tulipus* and the polychaete *Idanthyrsus cretus.*

The second part of the document covers the main results from the work with the mollusc *Eualetes tulipus.* Since each chapter in this part is focued on a different biological question, each of them is self explanatory and contains the main divisions that any reader could find in a scientific article (from abstract until conclusions), except references and appendices that are in the last part to make the document more fluid. Thus, chapter 3 includes results related to the taxonomy (traditional and molecular taxonomy) and general ecology of the species, while chapter 4 is more focued on the results from analyses and techniques performed to establish the potential use of the species as a proxy indicator of Sea Surface Temperature-SST and salinity in the TEP.

The third part includes only one chapter, chapter 5, and it is focus on the results related to the taxonomy, general ecology and the use of molecular tools to identify the population(s) of the polychaete *Idanthyrsus cretus* and their origin within the TEP. In the same way as in the second part, chapter 5 is self explanatory and contains the main divisions of any scientific article (from abstract until conclusions), except references and appendices.

The last part, part 4, comprises therefore the conclusions, appendices, and references (including web references) of the whole work.

PART 1. GENERAL THEORETICAL OVERVIEW

Chapter 2. Overview of the area, techniques and taxonomic groups of interest

2.1. Description of the Tropical Eastern Pacific-TEP:

The Tropical Eastern Pacific (TEP) ranges between latitudes 23º26'N in the Gulf of California (Mexico) and 1º28'S off south of Ecuador in the Galapagos Islands (Macintyre *et al.* 1992, Cortés 1997, Glynn and Mate 1997), and is classified as a distinct bio-geographic zone with unique flora and fauna (Figure 2). The coral reefs in this region are typically small fringing reefs (one to several hectares), shallow (<10 m deep), and species poor (no more than 23 species belonging to 8 genera in comparison with more than 500 species in, for example, the Western Pacific). Their typical structure is of a thin coral layer build with inherited relief from non-carbonate substrates. They are patchily distributed on both mainland shores and adjacent islands, and the largest accumulations occur only in near-shore areas protected from sporadic severe El Niño warming events and seasonal cool upwelling pulses (Glynn and Mate 1997, Macintyre *et al.* 1992, Diaz *et al.* 2000, Glynn 2001a). Two main types of corals reefs can be recognized in the TEP: pocilloporid reefs and poritid reefs (Cortés 1997).

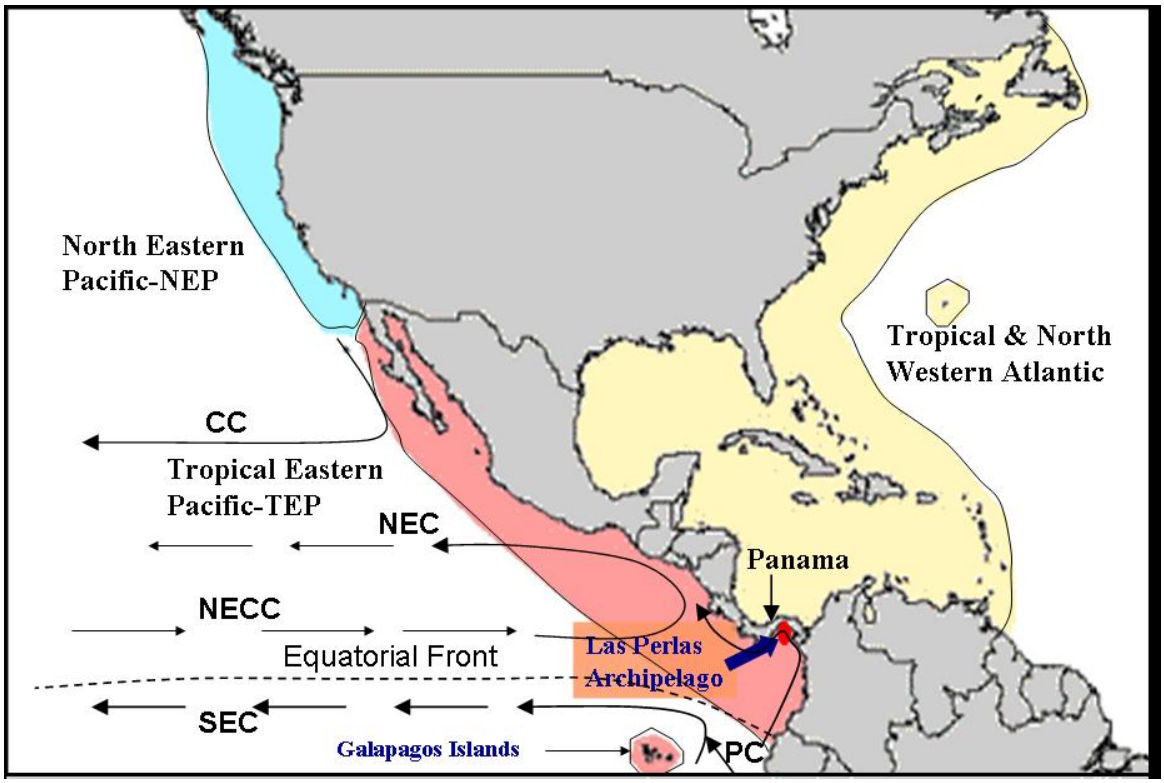

Figure 2. . The Tropical Eastern Pacific-TEP and the study area, the Las Perlas Archipelago. Modified from Glynn and Mate (1997) and http://www.reef.org/data/database.htm.

There are five dominant current systems in the TEP (see Figure 2): California Current-CC; North Equatorial Current-NEC; North Equatorial countercurrent-NECC; Peru Current-PC; and South Equatorial Current-SEC. The CC transports cool water (14º-20ºC) to the south, gradually warming as it reaches Baja California. Depending upon the season, the CC can join the NEC where it ranges between 25º-28ºC to move then in a westerly direction. The NECC, often strongest in August, flows from west to east across the Pacific. It originates west on the International Date Line, increasing in speed until it attains 30-40 cm/sec upon entering the TEP. This current system delivers water chiefly to Colombia, Panamá and Costa Rica, and seasonally reaches as far north as Clipperton (Baja California). It is suggested that the easterly flowing NECC makes landfall right at the TEP reefs sites of highest coral species diversity. Like the CC, the PC is initially cool (18º-22ºC) and warms as it joins the SEC, moving toward the equator and to the west. Within the region of reef development, the PC mainly influences the Ecuadorian coast and the Galapagos Islands (Glynn and Mate 1997).

The physical and oceanographic conditions of the region, such as extremes in temperature, salinity and nutrients (N and P) are adverse for the reef growth. The whole region has a very shallow thermocline that results in the intrusion of cold waters that reduce the tolerance of some marine animals to settle. Coastal upwelling, a product of the trade winds, is prevalent in the Gulf of Tehuantepec (Mexico), Gulf of Papagayo (Costa Rica), Gulf of Panama, Gorgona and Malpelo Islands (Colombia), and Galapagos Islands of shore Ecuador (Cortés 1997, Diaz *et al.* 2000). This upwelling brings cold (minimum of 14.7ºC) and nutrient rich sea water (and therefore high turbidity) that impacts the coral reef zones and associated fauna giving, as a result, smaller reefs, lower accumulation rates, thinner frameworks in terms of vertical growth, and slower coral growth rates than in non-upwelling areas such as the Gulf of Chiriquí, in Panamá. The upwelling brings, as a secondary effect, extensive algal growth, limiting coral recruitment and the expansion of living coral surfaces (Macintyre *et al.* 1992). The other thermal extreme is the normally high temperature during the non-upwelling season. This high temperature is pushed to the extremes (e.g. 30ºC) during strong El Niño years, causing widespread death of marine life. Sea Surface Temperatures-SST during the 1982-83 El Niño event were 3-4º above normal for 9 months off the Galápagos Islands, and 1-2º above normal during the same period off the coasts of Costa Rica and Panama. As a result, the coral mortality rates were close to 80% in Panamá and 97% in Galápagos (Macintyre *et al.* 1992). Large salinity fluctuations also

characterize sections of the TEP, as in the Panama Bight where the salinity decreases from 34‰ (dry season) to near 20‰ (wet season) due to high local rainfall. Extreme low tides (that leave the shallow reef flats aerially exposed) and phytoplankton blooms (red algal tides) have also caused noticeable coral mortality (Macintyre *et al.* 1992, Cortés 1997). Another physical impact is the reduction in light penetration by turbidity. The turbidity is caused by high nutrient waters that promote the phytoplankton blooms during the dry season (upwelling) and by the sediments released (siltation) with freshwater discharges from rivers due to erosion of adjacent land masses during the wet season (Macintyre *et al.* 1992, D´Croz and Robertson 1997).

There are also very complex interactions among the TEP biological communities. For example, asexual reproduction in the coral *Porites lobata* is aided by the actions of indirect predators like the fishes *Sufflaemen veres* and *Pseudobalistes naufragium*, and this appears to promote the survival of the coral species. High nutrients promote intense competition between algal turf and corals, in which usually the corals lose, thus asexual reproduction becomes important for growing and survival. *P. lobata* is typically infested by the boring bivalve *Lithophaga* spp, and the fishes, in their search for the bivalve, bite and remove coral fragments of 2-5cm diameter. These fragments are discharged by the fish and a high percent of them survive and grow adjacent to the parent colonies. Another complex interaction involves the corals *Pocillopora* spp which are commonly consumed by the sea star *Acanthaster planc*i. However *A. planci* never appears to reach outbreak levels in the TEP. The branched corals are defended directly from the sea star by crustacean guards (xanthid crabs and the harlequin shrimp *Hymenocera picta*) in a mutualistic relationship in which the crustaceans obtain protection from predators. The crustacean inflicts small linear wounds (<1cm) on the arms of the sea star. The wounds themselves are not fatal, but are used by amphinomid worms (*Pherecardia striata*) to enter the body cavity where they consume soft organs and eventually kill the sea star. Where both worms and crustaceans are abundant, there are no sea star outbreaks and thus this limits the sea star from reaching unprotected coral species (Cortés 1997). Bioerosion (biological destruction) has also been reported as being extremely high in the TEP reefs (especially after the 1982-83 EL NIÑO) in comparison with the low rate of recovery (resilience) recorded during the last 200 years before the 1982-83 El Niño and therefore seems to be slower than in other regions (Colgan 1989, Cortés 1997, Díaz *et al.* 2000). Recent studies indicate that bioerosion exceeds carbonate deposition, resulting in the slow erosion of reef structures. The initial perforation is caused by,

amongst other factors, moluscs, sipunculids, polychaetes, and sponges in order to obtain shelter or food, weakening the coral structure that is then bioeroded by sea urchins or fishes (Cortés 1997, Barrios and López-Victoria 2001, López-Victoria *et al.* 2006).

From the geological point of view, perhaps the most interesting event for the TEP is the emergence of the Isthmus of Panama some 3.2 to 3.5 million years ago and the disruption of the seaway that brought marked changes in the Middle American marine environments, affecting circulation, depth, temperature, sedimentation, nutrients and productivity. Before the emergence of the isthmus the TEP had experienced sporadic booms of reef building since the end of the Cretaceous (65 million years ago) and global collapse of reef buildings, until the closing of the isthmus. During that period there was open circulation between the TEP and the Tropical West Atlantic-TWA, and the TEP was the westernmost extension of the Tethys sea (that includes the current Mediterranean Sea) and therefore shared species with the Atlantic (Colgan 1989). Then, after the emergence of the isthmus, the biota of the TEP suffered subsequent regional extinction events and speciation due to the geographic isolation and divergence in environmental conditions (Glynn and Mate 1997, D'Croz and Robertson 1997). While the Tropical West Atlantic-TWA continued to be influenced by the Equatorial Atlantic Current, the TEP fell under the influence of seasonal colder currents flowing toward the equator from both North and South America, in synergic effect with seasonal wind-driven upwelling systems that developed at several sites along the coast of Central America (in the last 3.5 million years). In addition, there were warming events that potentially reduced live coral cover. Both, the warming and cooling events resulted in the extinction and later colonization of several coral species. As a result, while the new Caribbean Sea remained warm thought the year, the TEP became more seasonal and oceanic productivity and high availability of dissolved nutrients exerted an important influence on the coastal oceanography of the area. This large physical change seems to be responsible for the modifications of species in the TEP (Cortés 1997, D'Croz and Robertson 1997), with four main hypotheses: 1) TEP faunal composition is a product of migration from the Central Pacific, 2) TEP faunal composition is a result of speciation and extinctions (vicariance), 3) coral distribution patterns are ancient when compared with the age of the present, 4) the previous viewpoints are not mutually exclusive because all the hypotheses describe events that have actually taken place (Cortes 1997).

During the Cretaceous-oligocene time 36 coral genera existed, that then reduced to 18 genera during the Miocene, 8 during the Pliocene and Pleistocene, and then increased to 12 during the Holocene. Only 6 genera of those remain at the present (Colgan 1989, Cortés 1997). From the actual 23 species belonging to 8 genera, 12 are vulnerable to extinction because they occur as small, isolated populations. In the same way, 6 other species have suffered local or regional extinctions (e.g *Acropora valida* in Colombia, *Porites synaraea* in Costa Rica, *Millepora* spp in Galapagos) during the 1982-83 El Niño (Colgan 1989, Glynn 1997). The actual and dominant *Pocillopora damicornis* and *Porites lobata* reefs were established about 5,500 years B.P in the TEP coastline and flourished until 500 B.P., when changing river patterns and more recent deforestation of adjacent coast created siltation stress. In contrast, TEP reefs around offshore islands have only a thin (<1.0 m) *Pocillopora damicornis* framework cover, with a maximum age of 500-2,000 years B.P. Episodic strong El Niño warming events seem to be responsible, at least in part, for the widespread mortalities that have occurred since the emergence of the isthmus and have limited their Holocene development (Colgan 1989, Macintyre *et al.* 1992, Cortés 1997, Glynn and Mate 1997).

2.1.1. TEP coral reefs and Pocillopora type reefs:

There are several studies that provide information about the structure and state of Tropical Eastern Pacific-TEP coral reef environments (see Glynn and Mate 1997, Díaz *et al.* 2000, D´Croz and Mate 2000, Barrios and Lopez-Victoria 2001, D´Croz *et al.* 2001, Hueerkamp *et al.* 2001, D´Croz *et al.* 2003, Mate 2003a,b), and recently researchers have begun monitoring and carrying out historical analysis on coral organisms from that area (e.g. Glynn and D´Croz 1990, Guzmán *et al.* 1991, Guzmán and Jarvis 1996, D´Croz and Robertson 1997, Glynn *et al.* 2001, Garzón-Ferreira *et al.* 2002a). Such studies defined natural (i.e. temperature) and anthropogenic factors (i.e. pollution, heavy metals) that may be responsible for the general deterioration that is evident in some TEP coral reefs. Nevertheless, recent heightened effects of sea warming have increased the opinion that global climate change poses the greatest threat to coral reefs (Glynn *et al.* 2001). In response to the above, there is a small but growing number of works related to stable isotope profiles in other organisms, like molluscs (e.g. Jones *et al.* 1986, Romanck and Grossman 1989, Aharon 1991, Kennedy *et al.* 2001, Richardson 2001, Owen *et al.* 2002, Elliot *et al.* 2003).

2.1.2. Health and monitoring in the TEP coral reefs:

Monitoring is the gathering of data and information on a defined environment constantly and periodically on from a baseline study (Hill and Wilkinson 2004). There are actually 22 methodologies established for monitoring coral reef environments (Table 1). In general the methodologies can be organized into two basic categories in terms of information of the environment: 1) methodologies that focus on basic monitoring programmes (what is their actual structure, where are those reefs, how do they function now and in the future, reef health, etc, and are focused into community and management monitoring for MPA's); and, 2) more advance methodologies focused on the biology of the ecosystem or part of it (flux, nutrients, etc, and are part of research monitoring). They are addressing the next basic questions (Hill and Wilkinson 2004) that can be answered with an effective monitoring programme, which will include a number of monitoring methods:

-Are coral reefs healthy and improving?

-Are threats damaging coral or other organisms?

-Are fish populations increasing in a protected area?

-Has management action been successful?

-Are the economies of local communities maintained or improved?

-Do the communities understand the need for management and want to assist?

-Is tourism a positive or negative benefit for the coral reef area?

Table 1. Monitoring techniques and monitoring programmes used currently in Eastern Pacific coral reef environments. Modified from Hill and Wilkinson (2004) and Turgeon *et al.* 2002. Techniques: 1)Airborne and satellite digital remote sensing, 2)aquanaut survey, 3)Atlantic and Gulf rapid reef assessment-AGRRA and Rapid Ecological Assessment-REA, 4)bioindicators: butterfly-fish, 5)participatory coastal resource assessment, 6)recruitment surveys and recruitment plates, 7)reef check, 8)reef fish visual assessment and reef Roving diver technique, 9)sedimentation monitoring, 10) sea stewards, 11) stationary sampling technique, 12)tissue monitoring, 13)towing surveys: manta tows and towed-diver surveys, 14)transects: chain transect, 15)transect: line or point intercept transect, 16)transect: photo quadrats, 17)Transect: video transect, 18)coral density banding and isotopes, 19)spectroradiometry, 20)monitoring system of Colombian coral reefs-SIMAC (part of global coral reef monitoring program-GCRMN), 21)NOAA, 22)STRI. Category: 1) methodologies that focus on basic monitoring programmes (what is their actual structure, where are those reefs, how do they function now and in future, reef health, etc). 2) Methodologies that focus on the biology of the ecosystem or part of it (flux, nutrients, etc).

TECHNIQUE	ORGANISMS MONITORED	MAIN QUESTIONS	MAIN PARAMETERS	IMPACTS ASSESSED	METHOD. CATEGORY	PROGRAMME	REFERENCES.
1	Coral, algae, active sediment, antecedent fossil reef	Can spectral imagery be used for bathymetry, spatial and compositional reef distribution, bleaching?	Coral cover, geometry of sedimentary system, spp composition, water color, SST	Sediment cycles, global climate, interaction of geological and biological processes, disturbance, coral reef health/cover	1		Turgeon *et al.* 2002 Hill and Wilkinson 2004 Armstrong 1992 Maritorena 1992 McConnell and Best 1992 Nakamori and Sugai 1992 Ogden *et al.* 1992
2	Coral, fish, algae, marine invertebrates, substrate type, echinoderms	Community changes interannually, potential causes of major changes	% live/dead coral cover, % macroalgae, % coral damage	Local to global reef changes, coral reef health indicators	1	Part of GCRMN	Hill and Wilkinson 2004 Turgeon *et al.* 2002
3	Coral, fish, algae, echinoderm *Diadema* sp.	Spatial/temporal patterns in coral mortality, change in algal/herbivore and fish community	%coral cover, mortality, % algae, bite marks, key fish spp and abundance	Scale of mortality, patterns of size, spp, zones, spp declines, altered trophic webs	1	AGRRA, GCRMN	Hill and Wilkinson 2004 Brainard *et al.* 2005 Turgeon *et al.* 2002
4	Coral, fish, microatolls (disc shape coral colonies expose to low spring tides)	Changes in ecological conditions, situation of important demersal fisheries, geographical variations in environmental parameters	No. and size of fish territories, rates of agonistic and feeding behavior, % coral cover, fish spp and abundance, fish recruitment success/year	Anthropogenic effects on reefs, small to chronic reef disturbances, early warming, fisheries, fishing pressure, population dynamic, recruitment, monitoring of sea level change	1,2?	Part of REEF CHECK	Hill and Wilkinson 2004 Doherty *et al.* 1992 McManus *et al.* 1992 Smithers 1992 Turgeon *et al.* 2002

TECHNIQUE	ORGANISMS MONITORED	MAIN QUESTIONS	MAIN PARAMETERS	IMPACTS ASSESSED	METHOD. CATEGORY	PROGRAMME	REFERENCES.
5	Coral, fish, algae, invertebrates	Significant economic resources, resource use issues, dynamic of artisanal fishery, spatial and seasonal pattern of reef resources	Spatial substrate distribution, value/revenue of reef resources, fish spp and distribution, harvest effort/reef resource product.	Over fishing, destructive fishing, fishing by outsiders, other human impacts, cultural factors and fishery management	1	REEF CHECK	Hill and Wilkinson 2004 Turgeon *et al.* 2002
6	Coral, algae, other marine invertebrates	Distribution and abundance of corals and recruits, genetic relationships	Recruitment distribution and abundance, %adult coral cover	Are reefs self seeded?, stability of coral communities	1,2?	Part of GCRMN	Hill and Wilkinson 2004 Jaap *et al*. 1992 Brainard *et al.* 2005 Turgeon *et al.* 2002
7	Coral, fish, algae	Baseline information, annual change	%coral cover, species distribution	Human impacts, endemic species, economic bioindicators	1	REEF CHECK, GCRMN,	Hill and Wilkinson 2004 Smith and Homer 1992 Turgeon *et al.* 2002
8	Fish, spiny lobster	Spatial distribution of, temporal variation of, effects of refuges in reef fishes, storm effects	Abundance, diversity, biomas, spp richness, frequency of occurrence	Habitat and fish association, design and assessment of harvest refuges, fishing effects, physical impacts	1	Part of REEF CHECK	Hill and Wilkinson 2004 Turgeon *et al.* 2002
9	Sediments	Change in depth over time, change in composition and source	Sediment depth, sediment source, size distribution	Effects of reforestation on sedimentation of coral reefs	1	Part of GCRMN	Hill and Wilkinson 2004 Turgeon *et al.* 2002
10	Fish, sea urchins	Effect of no take zone on density of target spp	Sea urchin density and size, damselfish density	Over-fishing, effect of MPAs	1	Part of GCRMN, part of REEF CHECK	Hill and Wilkinson 2004 Turgeon *et al.* 2002
11	Fish	Habitat-fish associations, long-term variability	Fish No. and spp, species richness	Habitat change, coastal development	1,2?	Part of GCRMN, part of REEF CHECK	Hill and Wilkinson 2004 Turgeon *et al.* 2002
12	Coral	Natural variation, changes in health of corals	Proteins, carbohydrates, lipids, zooxantella density	Natural variation, bleaching, nutrients	1,2	NOAA	Hill and Wilkinson 2004 Miller and Hulbert 1992 Turgeon *et al.* 2002 www.coris.noaa.gov/data

TECHNIQUE	ORGANISMS MONITORED	MAIN QUESTIONS	MAIN PARAMETERS	IMPACTS ASSESSED	METHOD. CATEGORY	PROGRAMME	REFERENCES.
13	Coral, algae, fish, marine invertebrates, clam *Tridacna* sp, Urchins	Impact of marine invertebrates, current status of reefs, long-term decline of reefs	% coral cover, other benthos, % marine invertebrates, fish abundance	Destructive fishing, change in coral cover	1	Part of GCRMN NOAA	Hill and Wilkinson 2004 Brainard *et al.* 2005 Turgeon *et al.* 2002 Miller and Hulbert 1992 www.coris.noaa.gov/data
14	Coral	Estimate of spatial index of reef, change of dominant coral spp	% coral cover, rugosity, species diversity	Change in three dimensional cover	1,2?	As in AGRRA	Hill and Wilkinson 2004 Turgeon *et al.* 2002 Porter *et al.* 1992a Ohlhorst and Liddell 1992 Jaap *et al.* 1992
15	Coral, fish, algae, mobile invertebrates, sponges substrate, marine invertebrates	Reef recruitment and mortality, dynamics of community changes, reef zonation, effects of MPAs, effects of manmade vs. natural disturbances	Coral cover, spp diversity, coral colony size, partial mortality, % benthic cover, fish No., sediments	Pollutants, coral diseases, dredging, over fishing, natural disturbances, nutrient enrichment, sewage, sedimentation, bleaching, divers impact, baseline surveys	1,2?	Part of GCRMN, part of REEF CHECK, part of CARICOMP, Part of MBRS-SMP	Hill and Wilkinson 2004 Ohlhorst and Liddell 1992 Mendes 1992 Mundy and Babcock 1992 Turgeon *et al.* 2002
16	Coral, algae, fish	Dynamics of community change, baseline surveys, harvest refugee	% coral cover, spp diversity, spp richness, size class distribution, frequency	Pollution, sedimentation, fishing, alien spp, natural change	1,2?		Hill and Wilkinson 2004 Turgeon *et al.* 2002 Brainard *et al.* 2005 Jaap *et al.* 1992 Mendes 1992 Porter *et al.* 1992b
17	Coral, algae, fish, benthos	Effect of aquarium collection, nutrient effects on coral communities, human effects on changing reefs	Coral cover, spp diversity, spp density, algal cover, age classes	Pollution, sedimentation, aquarium collection, bleaching, diseases, vessel groundings, over-fishing	1,2?		Hill and Wilkinson 2004 Turgeon *et al.* 2002 Jaap *et al.* 1992 Carleton and Done 1992

TECHNIQUE	ORGANISMS MONITORED	MAIN QUESTIONS	MAIN PARAMETERS	IMPACTS ASSESSED	METHOD. CATEGORY	PROGRAMME	REFERENCES.
18	corals	Effect of temperature and environmental variables on coral growing, change in coral growth over time	Counting, appearance and timing of skeletal coral bands, oxygen isotopes, carbon isotopes	Seasonal coral growing, time series for coral growing, effect of temperature on coral growing	1		Barnes *et al.* 1992 Moore *et al.* 1992 Adkins *et al.* 2003
19	Water column	Can underwater spectral irradiance be used to reflect properties from bottom?, spatial and compositional reef distribution	Spectral irradiance from water column, reflection properties of substrates, light absorption in water column	Base line surveys,	1		Maritorena 1992
20	Coral, algae, sediments, clorophyll, marine invertebrates, fish	Community changes interannually, spatial/temporal patterns in coral reef structure and coral community/change in coral health	Coral cover, algal, other marine invertebrates cover, benthic spp composition, sea fans abundance, coral diseases, richness and abundance of reef fish, abundance of ecological or economical important marine invertebrates, temperature, sst, salinity, visibility, seston, sedimentation, chlorophyll	Local to regional change, coral reef health indicators, anthropogenic effects, sedimentation on coral reefs	1,2	GCRMN, CARICOMP, REEF CHECK	Garzón-Ferreira *et al.* 2002b

TECHNIQUE	ORGANISMS MONITORED	MAIN QUESTIONS	MAIN PARAMETERS	IMPACTS ASSESSED	METHOD. CATEGORY	PROGRAMME	REFERENCES.
21	Coral, algae, sediments, nutrients, chlorophyl, marine invertebrates, fish	Community changes interannually, spatial/temporal patterns in coral reef structure and coral community/change in coral health	Coral cover, algal, other invertebrate cover, benthic spp composition and abundance, fish spp and abundance, diseases and bleaching, coral damage, physical impacts, temperature, SST, salinity, nutrients, chlorophyll, oxygen, turbidity, sea level, sea circulation, coastal and marine pollution, tourism, fishing, trade of coral reef spp, invasive spp	Local to global climate change, coral reef health indicators, anthropogenic effects, sedimentation on coral reefs	1,2	GCRMN	www.noaa.gov/climate.html www.coris.noaa.gov/data Miller and Hulbert 1992 Brainard *et al.* 2005 Ray and Atwood 1992
22	Coral, algae, sediments, marine invertebrates, fish	Can ultraviolet light affect growth rate in reef organisms?	Mean daily spectral irradiance at 0, 1, 10 and 24m depths.	Calculation of cumulative dosage rates experienced by reef organisms.	1,2		Wellington and Gleason 1992

2.1.3. Importance of the TEP and justification of the work:

All the characteristics mentioned above for the TEP and its marine environments, make the region possibly one of the smallest natural marine laboratories in which to study community structure and function at a regional level. The TEP is not only a testing ground for biological theory but it is also a laboratory for paleoclimatic and oceanographic reconstruction (Cortés 1997). The proximity in time and space of TEP and Caribbean marine biotas (separated for only about 3.5 million years and 70 km respectively), also brings special relevance for inter-oceanic evolutionary studies and regional comparisons (Glynn and Mate 1997). Finally the presence of reefs in an area like the TEP of Panama, exposed to strong disturbances such as upwelling, ENSO, extreme low tidal exposures (up to 6m range), heavy sedimentation, and intensive biological interactions (amongst others), offers numerous opportunities for the study of conditions that limit development and growth of reef environments and their associated fauna. Recent findings suggest that ENSO activity may be stronger now than at any time over the past 150,000 years (Glynn 2001b) and therefore it is important that such impacts be carefully quantified and evaluated in order to gauge the potential persistence of marine environments like coral reefs in the TEP region. Apart from their biodiversity and scientific relevance, the coastal habitats are an important resource for the countries of the region in terms of fisheries, aquaculture, and tourism, and are the basis of the economy for the communities of local people who rely on healthy marine ecosystems.

The results from this research in the area will complement work being carried out by several other UK and international institutions on marine communities. In the case of the vermetids, the use of this new proxy indicator could be used as an alternative species for temporal/ecological analysis of environmental change in areas where there are fewer, more scattered and threatened massive corals (e.g. the TEP).

The work will enhance the co-operation between several UK and overseas institutions (STRI, Panama) and it is intended that the results obtained from the project will be published in the scientific literature to add to the sparse but growing knowledge of specific environmental impacts on Tropical Eastern Pacific-TEP marine environments. The research will produce valuable collated datasets of environmental variables from a major marine geographic region and relate this information to changes in structure of sabellarids reefs and vermetids associated with coral reef environments. Agreements

have been obtained from various international/regional databases (e.g. STRI, INVEMAR, INOCAR, Museum of Chicago) to access local information about Sabellaridae, Vermetidae, coral reef structure and temperature. On the basis of the results, recommendations have been made to enhance further research required in the management of the marine areas under study. Improved methods for taxonomy and population ecology of vermetids and sabellarids, as well as time-series analysis and proxy monitoring of environmental variables over an area of TEP, in the case of vermetid communities, will enhance the information available to marine managers and policy makers.

2.2. Study area: Las Perlas Archipelago (upwelling):

The Las Perlas Archipelago in the Tropical Eastern Pacific-TEP of Panama (Figure 3) is a very productive area due to the existence of upwelling (D´Croz *et al.* 2003). This upwelling brings cold and nutrient-rich waters to the shallow coastal area (D'Croz *et al.* 2003). However, a remarkable characteristic of the TEP is that concentrations of surface nutrients remain continuously high and are never completely depleted despite the maintenance of high phytoplankton productivity (Agujetas-Medrano 2004), creating as a result a high turbidity that causes a low biodiversity in some taxonomic groups like corals (Glynn and Mate 1997, D'Croz and Robertson 1997, Diaz *et al.* 2000), in contrast to the Tropical Western Pacific (Glynn 2001). The upwelling occurs each year during the dry season that lasts from January to April (D´Croz *et al.* 2001), with the coldest period in March (minimum 15 ºC, average 20ºC) and the warmest period (maximum 32ºC, average 28ºC) between May and July (D´Croz and Robertson 1997, http://striweb.si.edu/esp/physical_monitoring/download_orts.htm).

The upwelling occurs each year during the dry season, January–April (D´Croz *et al.* 2001), when the Northeast trade winds cross to the Pacific via the low terrain of the Isthmus of Central Panama, interrupting the dominant southeasterly trade winds, in what seems to be part of the effects of the migration of the Inter Tropical Convergence Zone-ITCZ towards the south. These winds from the Caribbean, called the Panameño jets, push surface water far offshore and affect the depth of the thermocline (Agujetas-Medrano 2004).

The first phase of the upwelling, which can be delayed and starts during February, represents a decrease below 25°C in SST recorded mainly at Las Perlas Archipelago and the Panama Bay (D'Croz and Robertson 1997, D'Croz *et al.* 2003). The most intense upwelling conditions are achieved during March, when the centre of the cold plume reaches Sea Surface Temperatures-SSTs between 21 and 23°C towards Colombian territorial waters. During April the upwelling conditions seem to retrain in the Panama Bight (South Panamanian and Colombian territorial waters), disappearing from the whole area during May (Agujetas-Medrano 2004).

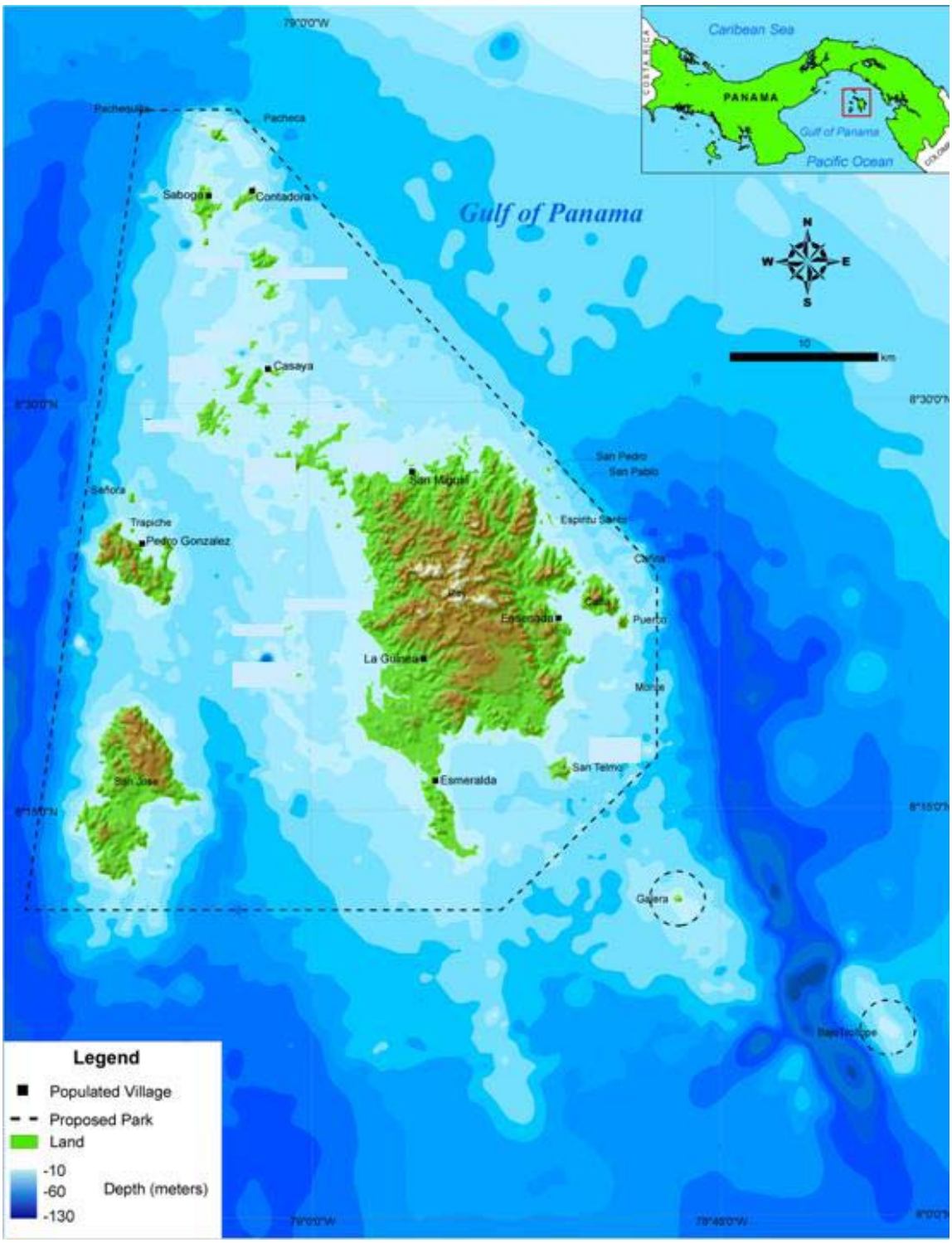

Figure 3. Study area: Las Perlas Archipelago. Modified from GIS-Darwin Project STRI-HW University (2006).

The upwelling effect, as well as phytoplankton biomass and nutrients, is remarkably reduced during the warm phase of the El Niño Southern Oscillation-ENSO (El Niño), in which there are higher SSTs. The opposite effect occurs during the La Niña period due to stronger wind transportation (Agujetas-Medrano 2004). The weakening in the upwelling conditions may occur one year before and after the ENSO events along the

Central American upwelling (Agujetas-Medrano 2004). The upwelling in the area and its effects on coral reef ecosystems are also mentioned by Glynn and Mate (1997).

2.3. Tools for time series analysis:

2.3.1. Chronology of calcium-carbonate marine organisms:

Marine lithologic indicators are organisms or rocks that can give an expression of certain features of a specific environment. There are examples for indicators of changes in environmental temperatures (ice sheets, reefs, carbonate platforms, molluscs, trees, kaolinite), anoxic environments (pyrite, black shales), or humid environments (coal) amongst others (http://earth.usc.edu/geol150/variability/proxy.html). Periodic growth structures (lines, bands, and rings) are preserved in the skeletons or hard parts of many organisms. Although the best known example is that of annual tree rings, periodic growth structures have also been long observed and studied in other organisms such as some corals or molluscs. The interest for this technique was initiated by Wells (1963, in Clausen 1974) when he described "daily" growth lines in living and fossil corals and used these in conjunction with annual growth structures to propose the growth-line method of "absolute" age-dating or geochronometry. Shortly thereafter, Wells' data were used in studies on the origin of the earth-moon system and the rotational history of the earth (Clausen 1974).

One of the main indicators of changes in water temperature is the reef environment. Tropical coral reefs grow mainly where the SST exceeds 18ºC, and optimal growth occurs at around 25ºC. At 25ºC the growth rate of massive corals is about 50% faster than at 18ºC. The dependence on temperature means a variation of growth rate with season. In the past there has been confusion in interpreting coral cross-sections, due to uncertainty about the process of coral growth. Some corals appeared to grow faster in the warmer wet season and others in the cooler dry season (Linacre and Geerts 1997).

Coral skeletons are composed of calcium carbonate ($CaCO_3$) in the form of aragonite. Each polyp, containing the living soft tissue of the organism, precipitates this mineral to form a protective skeleton. As the coral grows and adds new skeleton, the older skeleton remains as the foundation of the structure, creating an historical record of growth. In many organisms that grow throughout their lives, including coral, growth rates vary

depending on several conditions, such as nutrient availability and temperature. Sometimes these periods of alternating faster and slower growth produce banding inside the coral skeleton. X-ray examination in massive coral skeletons have shown layers of different density due to seasonal variations, like the annual rings of tree trunks (e.g. Guzmán and Cortés 1989, Charry *et al.* 2004). Charry *et al.* (2004) showed that high density bands (Figure 4) corresponded to dark bands in X-rays (wet season, warmer SST, and reproduction in Pacific of Panama) and low density bands to white bands (colder SST and dry season in the Pacific of Panama, also described in Guzmán and Cortés 1989). However other authors have found the same banding corresponding to the opposite conditions in temperature for other regions: warmer periods with white-low density bands (bleaching, growth diminished or dead when water becomes to warm) and colder periods with dark-high density bands (http://www.koshland-science-museum.org/exhibitgcc/historical11.jsp). Counting of the density layers in large colonies of coral provides therefore annual dating of the layers for several hundreds of years. Coral cores in the TEP have been dated back to 5,600 years with thickness ranges from 0.2 to 13.4m (Cortés 1997). New skeleton is not only added to the outside of the existing material, but also to a depth of several millimetres. So the process involves two aspects - the building of a scaffolding of skeleton and subsequent infilling of surface tissue. On the Great Barrier Reef-GBR the average growth rate is around 12 mm/year, varying between 5 and 30 mm/year (Linacre and Geerts 1997) while in the Las Perlas Archipelago (Pacific of Panama) the average growth rate for *Pocillopora* colonies is 23.76mm/year, with minimum and maximum values of 17.04-33.96mm/year (Glynn and Stewart 1973). Useful information about internal banding in corals and other organisms can also be found at the following webpages:
http://oceanexplorer.noaa.gov/explorations/03bump/logs/aug06/aug06.html,
http://www.azstarnet.com/clips/physics11a.html.

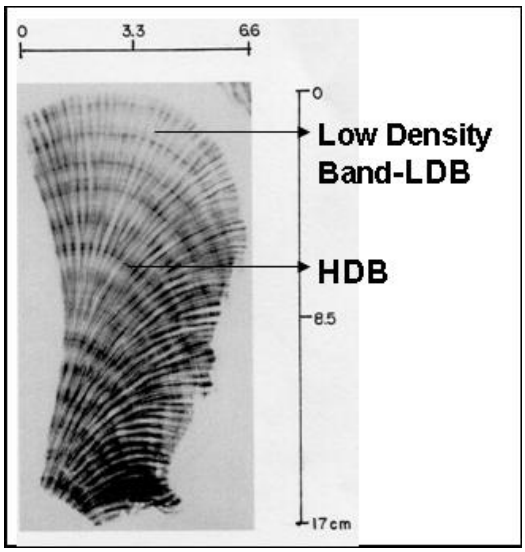

Figure 4. X-radiography positive print of the massive coral *Montastraea annularis* from Rosario Islands, Colombia. Modified from Charry *et al.* 2004.

A good example of the use of coral cores as indicators of changes in temperature has been evident for the GBR. There, the average annual calcification rate for ten very large coral colonies was relatively high during 1769-98 and 1940-1960, presumably indicating warm conditions, but low between 1819-30, indicating colder conditions. These ten coral cores were calibrated by matching with water temperature measurements since 1905. There appear to have been 12 major swings of around 0.6ºC below the present, during 1745-1905, i.e. roughly once every 14 years. The corals also indicate a warming of over 1ºC during 1979-1997 (Linacre and Geerts 1997). However the growth rate only gives an indication of temperatures when the temperature is a limiting factor (18-22ºC) and when other factors do not limit coral growth.

In order to grow a skeleton, corals utilize dissolved materials from the surrounding seawater. As the coral is producing its skeleton, it also incorporates other elements that may not be necessary to form aragonite. The abundance of some of these elements is a function of the environment surrounding the coral, and others are a function of metabolism. Sometimes it is a combination of both factors. If the elements are measured in the skeleton of a coral from the oldest parts and from those most recently produced, a picture of the variable chemical environment, and possibly the coral's life history, can be reconstructed because, if the elements vary seasonally in the water, they may also vary in the coral as it grows (Andrus 2003). There are then two more accurate methods to infer sea surface temperature variations from the annual growth layers of massive corals: oxygen isotopes and trace elements (Sr/Ca, Mg/Ca), which will be discussed in detail in later sections (See section 2.3.2, page 25).

Corals are one of the richest natural environmental recorders and archives because of the abundance of geochemical tracers, near perfect dating capacity, continuous growth of most species, the deposition of annual time markers in the form of skeletal density bands, and the fact that the species can live for very long periods of time. A 30cm-diameter coral colony growing at an average rate of 1cm/year will provide 20-25 years of baseline data, whereas massive colonies 3-6m high may provide data in the tropics related to centuries and recent palaeclimatology in relation to ENSO (Corroge *et al.* 2001). It is not unexpected that some reefs, especially those fringing heavily developed islands, are in serious decline, but what about reefs remote from direct human impacts? Finding them in decline is much more ominous, as it could signal widespread pollution, spreading epidemic diseases or the results of offshore overfishing.

The description above shows that assessing climate change (e.g. past magnitude of seasonal cycles and interannual variation) is a difficult task. Although this goal can be met for some tropical areas by making geochemical measurements on corals, work at higher latitudes has not generally been successful and very little is known about past climate variation on this timescale. That is the reason why researchers have begun to use other proxy indicators such as molluscs (Richardson 1988, 2001, Aharon 1991, Bemis and Geary 1996, Watanabe and Oba 1999, Owen *et al.* 2002, Silenzi *et al.* 2004).

The mollusc (Gastropoda:Vermetidae) *Eualetes tulipus* was specifically chosen for the study from Panama (described in this thesis) as a proxy species due to its characteristically long calcareous tube, which makes it easily distinguishable from other gastropods (Keen 1961, 1971, Rupert and Barnes 1994). Although its shell is formed from a mixture of calcium carbonate, as in corals, the calcium of the shell of molluscs is mixed with other protein material forming at least four layers. The outer periostracum is thin and composed of a horny protein material (conchiolon or conchin). The inner layers consist of calcium carbonate. The outermost calcareous layer is generally prismatic: mineral deposited as vertical crystals of aragonite surrounded by a thin protein matrix (Gregoire 1972). The inner calcareous layers (two or more) are laid down as aragonite (maybe sheets or lamellae) over a thin organic matrix (Rupert and Barnes 1994). The holotype and most accurate descriptions of the species are from Panama Bay (Keen 1971, 1982). At present, there is no published information on the growth rate of the tube in this group of molluscs.

2.3.2. Stable isotopes and trace elements:

Stable isotopes are atoms of the same element which have different numbers of neutrons and therefore different mass. However, because they have the same number of protons they have the same chemical characteristics. The difference in mass causes the isotopes to behave differently in nature. Oxygen has several isotopes, including $\delta^{16}O$ and $\delta^{18}O$. However, $\delta^{16}O$ is more abundant in nature (99.8% of all oxygen). To allow comparison with data from other works measurements of the $\delta^{18}O$/ $\delta^{16}O$ ratio in shells are reported using a special δ notation in which the value represents departures from a universally-agreed standard, in this case PDB (PDB-CO2, CO2 derived from PDB CaCO3 from the rostrum of a Cretaceous belemnite, *Belemnitella americana*, collected in the Pee Dee formation of South Carolina, international reference standard used to report variations in oxygen isotopes ratios). The carbonate standard, PDB, is no longer available, and the current international standard is NBS-19, a limestone sample that involves CO_2 with accepted $\delta^{13}C$ of +1.95 ‰ and $\delta^{18}O$ of -2.20 ‰ with respect to PDB (Francey and Allison 1998). As a result, $\delta^{18}O$ has a unit of part per thousand (per mil, ‰)

Oxygen isotopes are natural recorders of temperature and salinity. With respect to the temperature, the lighter $\delta^{16}O$ is more easily evaporated from the ocean and incorporated in water vapour. As a result, at low temperatures the ocean will have a larger abundance of the heavy isotope $\delta^{18}O$. When the temperature and kinetic energy increase, more of the heavy isotope $\delta^{18}O$ is evaporated. Therefore, at high temperatures more $\delta^{16}O$ is incorporated, proportionally, into the shells of organisms that secrete calcium carbonate, like *E. tulipus*. Thus, the ratio of $\delta^{16}O$ and $\delta^{18}O$ in the shell is systematically related to the temperature of shell formation. They are also recorders of salinity because both, $\delta^{18}O$/ $\delta^{16}O$, move in the atmosphere (air mass) from low to high latitudes. In the process the air loses water vapour as rain, including the heavy $\delta^{18}O$. Thus, the air mass and the ice sheet in the poles act as “storage areas” for the light isotope $\delta^{16}O$ (Stott 2007).

Isotopic and geochemical time-series generated from massive coral skeletons and mollusc shells are now being used to monitor environmental changes in the oceans and nearby coastal zones. They are also being used to reconstruct the past variations in tropical surface oceanographic conditions before any records were taken. Research has focused on the skeletal oxygen and carbon isotopic composition ($^{18}O/^{16}O$, $^{13}C/^{12}C$) as a

tracer of past oceanographic conditions. The health, diversity and extent of corals, and geochemical makeup of their skeletons, record a variety of other changes in the ocean surface water. These include temperature, salinity, fertility, insolation, precipitation, sea levels, storm incidence, river runoff, and human inputs (Aharon 1991, Bemis and Geary 1996, Richardson 2001, Owen *et al*. 2002).

Corals and molluscs produce annual growth bands, much like tree rings, which can also record monthly environmental changes. The $^{18}O/^{16}O$ ratio varies as a function of temperature and salinity. In general, higher ratios indicate colder temperatures, and low ratios indicate warmer conditions. Also trace element (Sr, Ca, Ba, Mg) concentrations offer a wider range of climatic interpretation by recording site-specific features, such as upwelling of cold deep water to the surface, runoff of freshwater from rivers, and mixing of different water masses. Radiocarbon (^{14}C) concentrations have been used to reconstruct ocean circulation, when deep water is brought to the surface the ^{14}C concentration in the coral skeleton is decreased (Macintyre and Smith 1974, Aharon 1991, Guzmán *et al.* 1991, Tudhope *et al.* 1995, 1996, 2001, Guzmán and Jarvis 1996, Bemis and Geary 1996, Guzman and Tudhope 1998, Fallon *et al.* 1999, 2002, 2003, Linsley *et al.* 2000, 2004, Cardinal *et al.* 2001, Richardson 2001, Owen *et al*. 2002, Swart and Grottoli 2003).

In studied organisms (e.g. Richardson 2001, Elliot *et al.* 2003) the oxygen isotopic composition of the skeletal material provides a robust recorder of water masses, recording the influence of both temperature and salinity. This tracer responds to water temperature through a temperature dependent fractionation (~-0.2 ‰ $\delta^{18}O$/ ºC), as well as to changes in rainfall, due to the influence of isotopically heavy ($\delta^{18}O$) rainfall on surface ocean composition and from river run-off which operates on a seasonal cycle. Since in tropical areas (e.g. TEP) SST and rainfall are intimately linked, the two factors combine to produce an enhanced environmental signal in the skeletal $\delta^{18}O$. According to Stott (2007) at an inter-annual time scale wet and warm conditions during the El Niño phase of the Southern Oscillation-ENSO will increase the deposition of isotopically light skeleton ($\delta^{16}O$) since heavy isotopes ($\delta^{18}O$) evaporate more easily. It has been proven with skeletal material from massive corals (e.g. McCulloch *et al.* 2003, Fallon *et al.* 2003), that Sr/Ca and Mg/Ca, can vary mainly as a function of temperature (about -0.7% Sr/Ca/ºC, and +3% Mg/Ca/ºC). Thus, these trace elements, used in combination

with $\delta^{18}O$, will enable the calculation of the SST and salinity contributions to the seasonal and ENSO cycles.

Barium has been used as a proxy of riverine sedimentary flux (Wan *et al.* 2005, McCulloch *et al*., 2003) and has been shown to reflect seasonal riverine discharges and upwelling conditions when measured in coral skeletons (Tudhope *et al.* 1996). Sediments suspended in river water contain barium (Ba), which is desorbed from the particles that carry it (clays) as they enter the ocean, where growing corals incorporate it into their skeletons along with calcium (Ca). Hence, when more sediments are carried to the sea by periodic flooding and more gradual longer-term changes in land use that lead to enhanced soil erosion, the resultant increases in sediment load are recorded in the Ba/Ca ratio of coral skeleton material in a proportional way (McCulloch *et al*., 2003). Thus, to obtain information related to the effect of sedimentary flux in marine communities, it is vital to bear in mind that inorganic P, Organic P and N, nitrite and nitrate are enriched in flood plumes resulting in higher levels of biological activity (e.g phytoplankton blooms or algal blooms), and that there are also physical effects such as increasing turbidity (McCulloch *et al*., 2003). Wan *et al.* (2005) showed that Ba/Ca ratios had seasonal cycles in an 8 year old coral (*Porites* sp), being 2-3 times higer during the raining summer season, except during the El Niño period of 1997-98. They also found that the main sedimentary fluxes that go into the South China Sea were provided by the Kaoping River, with 81% of the discharges in summer. They found that the seasonal cycles of the two records (records of riverine discharges and Ba/Ca concentrations in corals) were in perfect match except for the El Niño period. Tudhope *et al.* (1996) mentioned that the presence of distinct seasonality in the Ba records of corals and the coincidence of elevated Ba with the wet season indicates a strong environmental control on coralline Ba. Therefore, by measuring the Ba/Ca ratio, we can add a further important parameter to describe the environmental conditions associated with reef building in the TEP. Thus, these three element ratios selected (Sr/Ca, Mg/Ca, Ba/Ca) seem to give the best correlation with oxygen isotopes in order to eliminate noise when historical analysis of temperature is required, as has been confirmed in studies on massive corals and bivalves (e.g. Aharon 1991, Guzman and Jarvis 1996, Schrag 1999, Cardinal *et al.* 2001, Elliot *et al*. 2003, Fallon *et al*. 1999, 2002, 2003,).

In the case of the strontium/calcium (Sr/Ca) ratio, it is known that the coral absorbs less strontium when the temperature rises, and temperatures can be determined to within

0.5°C from the Sr/Ca ratio. This technique allows monthly 'mean' temperature anomaly estimates, from slices of coral corresponding to only a month's growth. The gradualness of ocean-temperature changes makes them less susceptible to the random disturbances which affect air and land temperatures, so underlying temperature trends are discerned more readily (Linacre and Geerts 1997).

A variety of methods have been used to determine Sr/Ca, Mg/Ca, and Ba/Ca ratios in both fossil and modern organisms, as a proxy for SST. These methods include x-ray analysis, Thermal Ionisation Mass Spectrometry-TIMS, Laser Ablation Inductively Coupled Plasma Mass Spectrometry LA-ICP-MS, Isotope Dilution Mass Spectrometry IDMS, and Inductively Coupled Plasma Atomic Emission Spectrometry ICP-AES, this last one is also called ICP-OES (Fallon *et al.* 1999, Tudhope 1999, Allison *et al.* 2001, Cardinal *et al.* 2001). In the case of the oxygen isotopes, the most common techniques are the isotope ratio Mass Spectrometry-MS and the Secondary Ion Mass Spectrometry-SIMS of the Ion Microprobe Facility-IMF (Tudhope 1999, www.geos.ed.ac.uk/facilities).

2.3.3. Temperature:

In the case of the Sea Surface Temperature- SST, global analyses of this parameter have been made at climate research centres (e.g. the UK Meteorological Office, National Oceanic and Atmospheric Administration-NOAA, or Meteo-France), internationally based on ship and buoy measurements, as well as satellite-derived data. Such analyses contain artefacts from the evolving mix of measuring methods and large errors over the extensive regions of the ocean that have been sparsely sampled. Therefore, existing SST analyses will be more persuasive if tested against a record that is sufficiently independent (http://www.geos.ed.ac.uk/research/globalchange/group1/#rem_ocean). Estimation of SST from some molluscs species have shown offsets from real values until 5°C (Owen *et al.* 2001). On the other hand, satellite models have different resolutions depending on the type of saltelite but, in general, are around 5km. Careful work over recent years on satellite systems and increase of in situ SST measurements to, at least, one measurement every 5° latitude-longitude cell has improve their precision to even 0.2km. However, comparisons of those models with records collected from organisms (e.g. corals or molluscs) could increase the precision of the satellite models to 0.1km, especially if the difference between the values collected from the organisms

and the real SST values are smaller than 5°C (Owen *et al.* 2001, http://www.geos.ed.ac.uk/research/globalchange/group1/#rem_ocean). In this study therefore the mollusc vermetid *Eualetes tulipus* is suggested as a new proxy tool for the recording of temperature and salinity in a geographical area (TEP) where it is only feasible to have occasional scattered recording stations. An example of this type of analysis in corals is the work of McNeil and Matear (2007), which can be found at http://web.maths.unsw.edu.au/~bmcneil/publications/McNeil%20and%20Matear,%202007.pdf.

2.3.4. Time series analysis:

A time series is an ordered sequence of values or collection of observations of a variable at equally spaced time intervals. The main applications of the time series are:

- To obtain an understanding of the underlying forces and structure that produced the observed data

-To fit a model and proceed to forecasting, monitoring or even feedback and feedforward control.

The special feature of the time series analysis is the fact that successive observations are usually not independent and that the analysis must take into account the time order of the observations. When successive observations are dependent, future values may be predicted from past observations. If a time series can be predicted "exactly", it is said to be deterministic. At the other extreme it would be stochastic, due to the fact that the future is only "partly" determined by past values. For stochastic series, exact predictions are impossible and must be replaced by the idea that the future values have a probability distribution, which is conditioned by knowledge of past values (Chatfield, 1975). There are many types of time series in the physical sciences, particularly in meteorology, marine science and geophysics. Examples in marine sciences are the analyses of water temperature or marine shells measured for a bay over 10 years, where the individual observations are averaged over periods of one month (Chatfield, 1975).

There are two general types of time series: continuous (continuous observations in time), and discrete (observations taken only at specific times, usually equally spaced). However, discrete time series can arise in several ways: to take equal intervals of time in a continuous time series to obtain a discrete time series (called sampled series), or to

accumulate values over equal intervals of time (e.g. rainfall measured daily) for those variables that do not have an instantaneous value (Chatfield, 1975).

It is also important to bear in mind the different types of variation that a time series could have in order to be able to deconstruct them into a trend, seasonal variation or other irregular fluctuations. There are four main types of variations (Chatfield, 1975):

a) Seasonal effects: time series with an annual variation.

b) Other cyclic changes: variations in a fixed period due to some other physical cause (e.g. daily variation in temperature).

c) Trend: defined as long term changes in the mean (e.g. increasing of mean Mg concentrations in organisms with life period). Here it is important to define what is meant by long term (e.g. several years).

d) Other irregular fluctuations: Times series in which there are random variations, even after trend and cyclic variations have been removed. In this case there are techniques to identify the apparently irregular variation and to explain it in terms of probability models such as moving average or autoregressive models.

Stationary time series:

A stationary series has the property that the mean, variance and autocorrelation structure do not change over time. Once a series is ploted in a graphic, the stationarity can be identified by the flat looking of the series. The above means a series without trend, with a constant variance over time, a constant autocorrelation structure over time and no periodic fluctuations or seasonality.

Most of the theories of time series analysis are related to stationary time series and for this reason the most of the analysis requires one to turn a non-stationary time series into a stationary one so as to use this theory. For example, the trend and seasonal effects could make a stationary time series look as a non-stationary (with systematic or cyclic changes), and therefore, it is essential to remove these type of effects from the data before trying to analyze the information.

The first step is to plot the observations against time to show up the most important properties of a series in terms of trends, seasonality, discontinuities, etc. If the series is approximately stationary, it will be useful to compute the mean and standard deviation of the observations.

Plotting the data may indicate if it is desirable to transform the values of the observed variable. The two main reasons for making a transformation are:

- To stabilize the variance: when the variance appears to increase because there is a trend in the series. Example: if the standard deviation is directly proportional to the mean, a logarithmic transformation is appropriate.
- To make the seasonal effect additive: when there is a trend and the seasonal effect appears to increase with the mean then it may be advisable to transform the data to make the seasonal effect constant (if the seasonal effect is directly proportional to the mean, then the seasonal effect is said to be multiplicative and a logarithmic transformation is required to make the effect additive).

2.3.5. Relationship of studied veriables and global warming:

The information above shows that growth increments in the skeleton, isotopic composition ($\delta^{16}O$, $\delta^{18}O$, $\delta^{13}C$, $\delta^{12}C$) and trace elements (Sr/Ca, Mg/Ca, Ba/Ca) are therefore useful biological data to corroborate the fast increments in SST during the last century (Macintyre and Smith 1974, Aharon 1991, Guzmán *et al.* 1991, Tudhope *et al.* 1995, 1996, 2001, Guzmán and Jarvis 1996, Bemis and Geary 1996, Guzmán and Tudhope 1998, Fallon *et al.* 1999, 2002, 2003, Linsley *et al.* 2000, 2004, Cardinal *et al.* 2001, Richardson 2001, Owen *et al*. 2002, Swart and Grottoli 2003) by using time series analysis techniques.

2.4. Population ecology through molecular analysis:

Molecular tools have diverse applications in marine ecology and have opened a new field of investigation that had stagnated in the absence of such tools. However, relatively few natural populations have been characterized due to the rather labour-intensive methodologies employed. In general, the techniques based on DNA sequence variation have practical advantages over allozyme techniques (protein electrophoresis). For example, Polymerase Chain Reaction-PCR allows assay of minute quantities of DNA that may come from ethanol preserved samples. However, the choice of any molecular methodology should be based on the expected efficiency of a given approach to a specific problem rather than the apparent sophistication of the method itself (Burton 1996). Molecular tools have been useful in examining the stability of populations though time and in assessing patterns of recruitment to geographically separated populations. The advance in these techniques will allow future work to employ the spatial and temporal field sampling required to monitor the impact of natural and anthropogenic changes in the environment (Burton 1996).

One of the main advantages of these techniques is the fact that the nature of the genetic "markers" themselves is often (but not always) unimportant, that different classes of genetic markers have different technical features making them more attractive for a specific study than other ones, and that they are able to assay smaller samples. The main disadvantages are that they are more expensive and usually more time consuming (Burton 1996).

PCR is used to greatly amplify the number of copies of specific fragments of DNA facilitating the subsequent analysis of the amplified segments. The primary requirement of using PCR resides in the development of short (e.g. 20 base pairs-bp) DNA primers complementary to the ends of the DNA sequence of interest. Although this suggests that it is necessary to know the DNA sequence under study, in fact this is frequently not true because high levels of DNA sequences are conserved between near taxonomic groups in numerous places in the genome. This allows the development of "universal primers" (that is gene-specific primers) that allow PCR amplification of DNA from a broad array of taxa. It is also possible to obtain specific DNA sequences from tissues of unknown origin (sequencing) and, by comparison to DNA sequences databases, determine the tissue source from within a phylogenetic tree of life, but this is a more complex and

time-consuming method (Burton 1996). The general steps in such molecular work involve: 1) DNA extraction, 2) PCR using universal primers, 3) agarose gel verification of PCR products (UV cameras) plus ultrafiltration purification of products, 4) DNA sequencing reaction, 5) polyacrylamide gel electrophoretic analysis of sequencing reaction products (automated sequencer), 6) comparison using a publicly accessible BLAST web like the GENEbank (Burton 1996).

PCR-based methods with arbitrary primers, called Random Amplified Polymorphic DNA-RAPDs techniques, have found increasing applicability in studies of mating systems and clonal structures in asexual organisms, identification of planktonic larvae and elucidation of temporal population stability and recruitment processes, and in general identification of marine invertebrates, fish and their larvae. The RAPDs technique has the advantage that it can be used in the absence of DNA sequence information (Burton 1996) and therefore is very useful to identify populations of a species for which there is minimal knowledge. Some of the advantages of PCR-RAPDs for population analysis (Hillis *et al.* 1996, Ferreira and Grattapaglia 1998) include:

- Only one primer maximizes number of bands generated per reaction (segments).
- It is recommended to use enzymes that recognize 4bp (e.g EcoRI, HindII) because they detect polymorphism in segments catalogued as monomorphic by other enzymes.
- To cut the DNA before the RAPDs simplify the process.
- To avoid ramping time or big temperature changes in short time during PCR will increase options to find and amplify even the less common segments.
- Knowledge of the mode of inheritance is critical in RAPDs (dominantly expressed). Without this information it may be impossible to distinguish alleles of a single co-dominant locus from independent products of non-homologus loci.

Therefore it is essential to examine heritability using appropriately designed crossing experiments, such as these two options:

- the **co-dominancy** and **homology** between segments of similar length can be confirmed by using the RAPD segment as a hybridization probe in the unknown segment.
- A combination of RAPDs and Single-Strand Conformation Polymorphism-SSCP could improve results in terms of **repeatability**. The nature of changes

detected by SSCP makes strict adherence to specific environmental parameters (e.g T^o, gel conditions) essential for consistent resolution.

- Sequencing is the best complementary technique to confirm results of RAPDs in terms of population analysis.

In Table 2 a comparison between the main techniques used for intra-specific analysis (population analysis) is given. The advantage of RAPDs in terms of time and cost, used in combination with sequencing, was the reason why this technique was chosen for the analysis of the *Idanthyrsus cretus* (Sabellaridae) population of Las Perlas Archipelago.

Table 2. Comparison between the main techniques used for intra-specific analysis (population analysis). Modified from Hillis *et al.* (1996), and Ferreira and Grattapaglia (1998). Highlights represent the main disadvantages and underlining the main advantages of each technique.

FEATURE	RFLPs	**RAPDs**	MICROSATELITES
Genetic expression	Co-dominant	dominant	Co-dominant
Number of alleles per locus	2-4 alleles	2 alleles	Multi-allelic
Range of fragment size analyzed	-With digestion/hybridization= >200bp -With PCR= 50ca-3000bp	500-3000bp (complementary sequence to primer in distances not bigger than 4000bp)	100-600bp
Availability of markers in genome	Almost unlimited	Almost unlimited	Almost unlimited
Distribution in genome	Regions with low number of copies	randomly	randomly
Steps in detection	+9 steps: e.g. 1)purified organellar DNA-expensive, 2) DNA-extraction by Phenol chloroform or kit, 3) digestion, 4) electrophoresis in agarose or polyacrilamide, 5) membrane transference, 6) cloning, 7)purification, 8) radioactive markers, 9) detection by hybridization with labeled probe (or Staining labeling with Ethidium Bromide-EB when combined with PCR), 10)moisturizing, 11) x-ray	4 steps: 1)DNA extraction by Phenol chloroform or kit, 2) denature and amplification by PCR, 3) electrophoresis in agarose, 4) detection by uv in gels with ethidium bromide and hibriditation (or restricting gel-isolated fragments or Single-Strand Conformation Polymorphism-SSCP) for calculation of band frequencies, 5)comparison by software gel-compare	4 steps: 1)DNA extraction by Phenol chloroform or kit, 2)denature and amplification by PCR, 3)electrophoresis in polyacrylamide, 4) detection with 5' label or incorporation of ^{32}p, 5)cloning
Preliminary basic information	2 (Genomic library, adequate probe)	2 (Basic primers, selected primers for the study)	5 (Genomic library, probes solution with microsatelites, DNA sequencing, basic primers, specific primers)
Efficiency	- LOW with digestion/hybridization (production of probes) and MODERATE for routine - HIGH with PCR (specific primers)	HIGH in routine	LOW in production of markers HIGH in routine
Sensitivity	- MODERATE-HIGH with digestion/hybridization -HIGH with PCR (specific primers)	HIGH	HIGH
Cost	- MODERATE-HIGH with digestion/hybridization -LOW With PCR (specific primers)	LOW	MODERATE
Genentic identification	MODERATE	HIGH	HIGH

FEATURE	RFLPs	**RAPDs**	MICROSATELITES
Advantages	-Potentially cover all genome according to available Genomic library -One population is enough to analyze a large number of characters -they are co-dominant: they express homozygote and heterozygote characters -the DNA used as a probe can be randomly cloned -Due to high DNA stability they can be preserved for use later	-faster and simpler than others (less reagents, materials, steps, etc). -10 times more efficient in time and people needed. -it is not essential to have a genomic library (it can use shorter primers with unknown or random sequences). -RAPDs can survey repetitive DNA regions. Because it is based on PCR it is more sensitive to detect polymorphism at DNA level. It is not necessary to have previous experience in molecular biology to use it.	-they posses the highest polymorphism information content. Therefore, any segregated population can be used as a reference for mapping.
Disadvantages	- too many steps -lack of genomic library -to much time (to obtain probes) before the real analysis -it needs trained technicians and adequate installations for radioactive disposal.	-Because the genetic expression is only dominant (band in gel when dominant gen present) you can not see differences between homozygote-AA or heterozygote-Aa (both are a present band), neither absence of band or large mutations (insertion, deletion, new restriction site, duplication, inversion, or large base substitutions). It needs to run several analyses to identify differences. -Low level of information per locus. -the lack of knowledge about the RAPD band used. -Some bands could easily be identified but not others because: 1-the selected primer has not the power to discriminate between amplification sites, 2- there is competition between amplification sites for substrate and enzyme, and 3-of changes in temperature and laboratory environment.	-Null alleles may fail to amplify because of large increase in size of product or mutations in primer sites. This is a potential problem because heterozygotes for null alleles will appear as homozygotes, changing estimates of genotype and allele frequencies. It can be more frequent when using primers designed from different species. -It is expensive, time consuming, and it needs trained technicians and adequate installations.
Use	-comparative mapping (mating systems) -Biogeography	-Genotype identification -Genetic analysis for species from which there is no previous knowledge (genotypes identification and genomic fingerprints). -population analysis (it detects differences of even only 1bp, for genetic diversity analysis in natural populations). -Mating systems -Hybrid zones and species boundaries.	-Genotypes identification -genetic mapping -Mating systems -Intraspecific diversity --Parentage -Relatedness

2.5. Groups of interest: Vermetidae and Sabellaridae:

2.5.1. Vermetidae:

The species under study are sessile and are best known as worm shells. They are part of a small number of gastropods adapted for a sessile existence. The worm shells, members of three unrelated mesogastropod families (Vermetidae, Turitellidae, and Siliquaridae), have typical larval and juvenile shells; but as the animal grows older, the whorls become completely separated, and the adult shell looks like a corkscrew or is completely irregular. Worm shells live attached to sponges, to other shells, or to rocks, and the separated whorls provide greater surface area for attachment. The foot is reduced, but an operculum is present (Rupert and Barnes 1994).

The shell is composed mainly of carbonate of lime and several other minerals like carbonates (aragonite, calcite), phosphates and oxides (magnetite, goethite, and aragonite). The shell itself is generally composed of one external layer (periostracum composed of conchiolon or conchin) and two or more inner calcareous layers, of which the centre layer is the thickest (e.g. aragonite) and the outer layer the thinnest (e.g. vertical crystals of calcite surrounded by a thin protein matrix). It is the mantle of the animal which is responsible for depositing the shelly matter, while the mantle margin carries the colour-depositing cells (Cernohorsky 1967). The typical gastropod shell is a conical spire composed of tubular whorls and containing the visceral mass of the animal. Starting at the apex, which contains the smallest and oldest whorls, successively larger whorls are coiled about a central axis, called columella, until the aperture from which the head and foot of the animal protrude. The shell may display a right-handed spiral or, as in the case of the vermetid species, a left-handed spiral (Rupert and Barnes 1994).

The species *Eualetes tulipus* appears to be a filter-feeder (deposit and suspension feeder) since a mucus bolus is produced following particle capture. Barnes (1974) mentions that some of the sessile Mesogastropoda (e.g family Vermetidae) are nominated as collectors of organic deposits or plankton. In these, as well as in other herbivorous species, the esophagus is modified as a crop and gizzard. The gizzard may be lined with cuticle or contain sand grains. Amylases and cellulases are produced by the esophageal glands or the digestive glands. The family could be also carnivorous

(planktonic hunters). The most common adaptation of carnivorous prosobranch is a highly extensible proboscis, which enables the snail to reach and penetrate vulnerable areas of the prey. The proboscis contains the esophagus, bucal cavity, radula and, at the tip, the true mouth. It lies within a proboscis sac which has a mouth-like opening to the outside (Cernohorsky 1967, Graham 1971).

A small number of prosobranchs are hermaphroditic (Cernohorsky 1967). In the case of sessile shells (e.g Vermetidae) the individuals tend to live stacked up on one another. The right shell margins are adjacent, thereby permitting the penis of the upper individual to reach the female gonopore of the individual below. Young specimens are always male. The sex of each individual is influenced at least partly by the sex ratio of the association. An older male will remain male longer if it is attached to a female. The scarcity of females influences certain of the males to become females, but once it becomes female it remains in that state (Rupert and Barnes 1994).

According to Keen (1971), the family Vermetidae is a Prosobranchia (subclass Mesogastropoda), part of the superfamily Turriteliacea (low to high spired sturdy shells with several whorls, a round aperture, and a multi-spiral horny operculum). The family (worm gastropods) is among the most puzzling of shells, and authors have confused them at every classification level from species through genus and family to phylum. Many tube-building annelid worms have been described as vermetid gastropods and vice versa. However the distinction between this family and the annelid worms is clear-cut: the gastropods have a three-layered shell that is glossy within and that begins with a tightly and spirally coiled embryonic shell, whereas the tube-building polychaete worms have a two-layered shell that is dull-surfaced within and that begins with a single tubular chamber. The family also differs from species of the family Vermiculariinae (another family from the superfamily Turriteliacea according to Keen 1971) because the nuclear whorls are different. Whilst the species of Vermiculariinae continue the regular spiral Turritella-like coiling and gradually become lax or unwound, in Vermetidae the nuclear whorls (those that the juvenile snail has when it emerges from the egg capsule) are two or three in number, with an aperture twisted forward and upward. Immediately upon emergence from the protection of the parent's tubular shell, the young vermetid finds a suitable place for settlement and attaches the shell to the substrate, beginning to coil its adult whorls at a right angle to the nuclear whorls.

2.5.2. Sabellaridae:

Species of the family Sabellaridae (which includes the species under study, *Idanthyrsus cretus*), also called honeycomb worms, build distinctive hard tubes from sand or other coarse particles and are commonly seen forming colonies on rocky surfaces in intertidial marine environments. They are also found on hard substrates down to depths of 6,000m. While some taxa are solitary, others like *Idanthyrsus cretus*, *Sabellaria* or *Phragmatopoma* are gregarious, forming extensive reefs that extend for kilometres. Sabellaridae are generally moderate in size, with adults in most taxa reaching between 20-50mm in length and having around 100 segments. When alive they are quite colorful, and often have a dark reddish or chocolate dorsum anteriorly with the remainder of the body being tan in colour or iridescent. The branchiae are usually bright red (Rouse and Pleijel 2001).

The sabellarids are easily distinguished from other polychaetes by having an operculum that is developed from anterior segments. The operculum bears one to three rows of large golden paleae on each side and these may form a complete crown, blocking the tube opening when the worm retracts. The head region is morphologically complex and there are several studies that attempt to determine its ontogeny and composition (e.g. Orrhage 1978 in Rouse and Pleijel 2001). These studies have allowed a reasonable assessment of the head but questions remain. There is also some confusion over the definition of prostomium and peristomium and the nature of appendages associated with the head (Rouse and Pleijel 2001). In addition to the golden paleae, the operculum of some taxa (e.g. *Idanthyrsus*) also bears nuchal hooks or spines that also come from the median chaetigerous sacs of the operculum, which would mean they are derived from the first anterior segment. Numerous oral filaments may line the inner margin of the opercular lobes and these are used in feeding. In addition to the head there are other four body regions: Thorax, parathorax, abdomen and caudal region. The thorax is referred to as the two segments immediately behind the mouth that bear neuropodia with capillary chaetae. Behind the thoracic segments are found either three (e.g. *Idanthyrsus*) or four (e.g. *Phalacrostemma*) parathoracic segments. The parathoracic sement are biramous and have lobate parapodia with sheaves of oar-shaped chaetae and companion capillary chaetae in both rami. The ventral surfaces of these segments have glandular elements used in association with the building organ for tube construction. The abdominal region comprises the majority of the body and is clearly distinguishable from the parathoracic region by an abrupt change in chaetal components. It has numerous uncini in single

rows. The body terminates with the caudal region, an apparently unsegmented (because in fact can have between 50-60 segments), smooth, cylindrical tube that in life lies recurved along the ventral surface of the abdomen. It has blood vessels and the gut travels along it and terminates at the anus (Rouse and Pleijel 2001).

Most of Sabellaridae are sessile and they are best known for the reefs they can form in intertidal to slightly subtidal areas. They prefer to live in areas of high wave action or current that can provide them with plentiful food and sediment for tube building. Large colonies can develop and disappear very quickly. Individuals can live more than 9 years but most of the species live for 3-5 years. Population densities of gregarious Sabellaridae can reach up to 60,000m^2 (Rouse and Pleijel 2001). Adult animal cannot survive outside their tube and are unable to replace an entire tube once it has been destroyed (Kirtley 1994)

Adult Sabellaridae have no bucal organ. The gut is a straight tube, though in the anterior abdominal region there is a thickened structure referred to as a proventriculus. They appear to be filter-feeders, using their oral filaments to generate a feeding current to capture fine particles and produce a mucus bolus. Large particles are captured by direct manipulation with the oral filaments or the pair of grooved palps and are eaten, ejected or used for tube building (Rouse and Pleijel 2001).

With respect to their reproductive system, they appear to be gonochoric, broadcast spawners and produce planktotrophic larvae. Gametes are located throughout the coelom of the abdominal region. Males are whitish in the lateral abdominal region whereas females are orange or purplish. Sperm have spherical heads typical of external fertilizers, but have very elongate acrosomes (Rouse and Pleijel 2001). The larvae can stay in plankton for extended period before settling and have long bundles of provisional chaetae, which appear to be anti-predation devices (Rouse and Pleijel 2001).

According to Kirtley (1994) *Idanthyrsus cretus* is part of the subfamily *Sabellariinae* because it present 3 parathoracic segments and, it is part of the genera *Idanthyrsus* because of the presence of a crown of unfolded chaetae.

PART 2. VERMETIDS

Chapter 3. Taxonomy and ecology of vermetids from Las Perlas Archipelago, Pacific Coast of Panama.

3.1. Abstract:

The mollusc *Eualetes tulipus* (Family Vermetidae) can be found in several areas within the TEP, including Panama, where it is associated with widespread reefs of branching corals (*Pocillopora*) and rocky areas. A new morphotype of *E. tulipus* is described associated with coral reef environments in Las Perlas Archipelago and other coral reefs in the TEP. The species showed an annual growth rate of 24mm with large variations among stations. These variations seem to be the response of the species to the surrounding environment, especially to the competition with the coral species that create the reef frame. Annual, monthly (spring tide bands) and daily growth bands were identified. The monthly bands showed to be organized in two different groups: small and compact spring tide bands, related to upwelling events, and wider spring tide bands produced by the mollusc during the rest of the year. The differences in the size of the annual growth bands may be a result of environmental factors that are affected in a larger scale, such as El Niño-ENSO. In terms of microhabitat, PCA analysis confirmed the requirements of the mollusc for for areas with high cover (>80%) and high density of branched corals and a minimal vertical growth of coral (>40 cm) that bring good protection against predators and a direct surface to the water column so they can access food. In terms of their general environment (coral reefs), PCA showed that coral cover can be lower than in microhabitat (> 60%) and the frame could include other benthic components apart from the branched corals. The only requirement was to be a proper reef framework, with a minimal vertical growth of 40cm so as to permit good protection to the vermetid. Beside, the distribution pattern observed in Las perlas Archipelago was, according to Poisson frequency and posterior chi-square test, the result of a random availability of clear space for settlement and a tendency to solitary life. In this way, the general requirements of the species was the same no mater what the environment was, i.e. rocky or coral reef areas.

3.2. Introduction:

The mollusc *Eualetes tulipus* (Chenu, 1843, ex Rousseau, MS) (Prosobranchia, Mesogastropoda, Vermetidae) was first described by Rousseau for Panamanian rocky areas as *Vermetus panamensis* (Adamas 1852, see *Vermetus panamenis* Rouseau in Chenu 1843) and by Chenu as *V. effuses* Chenu 1844, ex Valenciennes, MS (Keen 1971). Mörch (1861) also mentions the species *Vermetus panamensis*, Rousseau, Chenu 1843 and includes a variety '*tulipa'* with a diameter of aperture of 12mm and longitudinal axis of 65mm. He then describes a second species as *Vermetus tulipa*, Rouseau, Chenu 1843 from the Gulf of California and includes the variety *bridgesii* for the area of Panama. He describe the variety *bridgesii* (from *Vermetus tulipa*) as very similar to the previous variety *tulipa* (from *Vermetus panamensis*), but the colour in *bridgesii* is much obliterated and the last whorl is partly free and erect.

In 1961, Myra Keen described the new genus *Tripsycha* as similar to *Serpulorbis* but "*with an operculum, with medial and later whorls mostly unattached in a coiling cone form (coiled portions lacking feeding-tube scars), and last whorl lax and uncoiled. Nuclear whorls of two to four turns. The operculum nearly as large as the aperture, multispiral, slightly concave, with an appressed spiral lamina of several volutions. Mainly solitary shells but when crowded they may form a radial cluster of tubes due to the tendency of each individual to grow away from any near competitor*". The type species is *Vermetus tripsycha* Pilsbry and Lowe, 1932 that she assigns as *Tripsycha tripsycha* with a distribution from the Gulf of California to Acapulco, Mexico (Keen 1961). In 1971 the author described the subgenus *Eualetes* for the genus *Tripsycha* as shells of moderate to large size, with two or more nuclear whorls, operculum as large as aperture, concave, brown, with six or more volutions. In the subgenus she includes two species: *T. (Eualetes) centiquadra* and *T. (Eualetes) tulipu*s (Keen 1971). Finally, in 1982 Keen changed the subgenus *Eualetes* to genus status, with two species: *Eualetes centiquadrus* (Valenciennes 1846) from Puerto Penasco to El Salvador and *Eualetes tulipus* (Chenu 1843) for Panama Bay, both in the Tropical Eastern Pacific-TEP (Keen, 1982).

E. tulipus (Chenu 1843, ex Rouseau, MS) is described as: a shell firmly attached to substrate throughout life, with the last whorl not becoming disjunct or lax. Shell colur is ivory white to light brown, often with variegations or blotches of colur. Margins of coils markedly appressed, surface of whorls nearly smooth, not punctuate. Nuclear whorls

relatively small, nearly globose. Operculum is large, of several whorls, concave, thin, and brown. Diameter of aperture of large specimens is around 16mm, and the length of a coiled specimen is about 60mm. The specimens tend to be solitary rather than colonial. Numerous specimens were collected from Panama Bay (Keen 1971). For taxonomy of the family see chapter 2.

The family Vermetidae had two possible genera for the species found in Las Perlas (Keen and Coan 1974): *Petaloconchus* (that in Keen 1971 is mixed with the genus Tripsycha) and *Serpulorbis*. However the latter genus was discarded since the specimens collected in the current study of Las Perlas were compared with *Serpulorbis oryzata*, the only species of the three possible for this genus (*S. eruciformis*, *S. margaritaceus*, *S. oryzata*), that had similar characteristics to the current specimens in terms of distribution (TEP, including Panama), tube length (up to 250 mm), and type of coiling (coiled while attached, then is almost straight). It was concluded that the shells of the current specimens were completely different. For comparison, the holotype of *Serpulorbis oryzata* from the Natural History Museum of London was studied.

The molluscan species of this current study belongs to a group that is sessile and are best known as 'worm shells'. They are part of a small number of gastropods adapted for a sessile existence. The worm shells, members of three unrelated mesogastropod families (Vermetidae, Turitellidae, and Siliquaridae), have typical larval and juvenile shells; but as the animal grows older, the whorls become completely separated, and the adult shell looks like a corkscrew or is completely irregular. Worm shells live attached to sponges, to other shells, or to rocks, and the separated whorls provide greater surface area for attachment. The foot is reduced, but an operculum is present (Rupert and Barnes 1994). For details about nutrition and reproduction see section 2.5.1 (page 37).

Locality details of *E. tulipus*, patterns of geographical distribution, conditions for settlement, microhabitat, general environment and other biological and ecological details (nutrition, excretion and water balance, reproduction, larvae, nervous system and sense organs) are largely unknown as they were not recorded by previous authors. The only information is of that collected generally for the Class Gastropoda (e.g. Rupert and Barnes 1994). Descriptions by Keen (1971) and Keen and Coan (1974) mention the species as sessile on rocky areas in Panama Bay and associated with *Stephonoma*

pennatum. Mörch (1861) and Adams (1852) only mention it as an inhabitant of the Panamanian area.

With respect to ecological distribution patterns, the most common method to describe information related to such distribution (which has been accessed through counts of observations as in the case of these vermetids) is the Poisson distribution or Law of small probabilities. This statistic applies to counts of objects or events that are randomly distributed in area, volume or time, and where a large number of them are "at risk", but only a small proportion is actually captured within each observational unit of area, volume or time (Wardlaw 2001).

The aim of this work was therefore to describe the general taxonomy of the species (including confirmation of the new morphotype with particularly long tube-shaped shell by traditional and molecular analysis) and ecological aspects related to microhabitat and structure of the associated environment in the TEP coast of Panama. The main objectives developed during the work were:
1) To confirm the taxonomic group of this new morphotype as *E. tulipus* by traditional and molecular analysis and; 2) To describe its general ecological aspects e.g: a) growth bands, b) growth rate, c) microhabitat, d) structure of associated environment in terms of reef complexity and e) current reef structure and, f) the vermetids' distribution pattern.

3.3. Study area:

For description of the TEP and Las Perlas Archipelago, in the Pacific of Panama, see chapter 2.

3.4. Methodology:

3.4.1. Field work:

Primary historical information was obtained from shells and organisms collected mainly from Pedro Gonzales Island (station 55), and from another three coral reef environments in 2-7m water depth (stations 1, 12, 29). At each station (Figure 5, Table 3) at least one live organism with its shell was collected (tubes 15cm or longer, in which shell growth was parallel to the branches of coral to which it was attached) for taxonomic

identification (objective 1) and growth band analyses (objective 2a). All the analyses were performed in Scotland and confirmation of the specie was developed in USA.

Table 3. General information of field stations in Las Perlas Archipelago.

STATION.	NAME	LAT	LONG	DATE	AVERAGE DEPTH (m)
1	PACHEQUILLA	08°40'17.1"N	79°03'38.4"W	20/08/2004	6.6
3	PACHECA EAST	08°39.977'N	79°02.882'W	18/05/2005	5.4
9	SABOGA NE	08°37'40"N	79°03'13"W	12/08/2004	4.5
12	CONTADORA	08°38'02"N	79°02'09"W	17-18/08/2004	6.6
18	CHAPERA	8°35.537'N	79°01'.166''W	18/05/2005	4.8
21	MOGO-MOGO	8°33'51.6"N	79°01'13.1''W	01-17/05/2005	3.6
23	MOGO-MOGO	8°33'47.6"N	79°01'24.5''W	12/05/2005	3.9
29	SAN PEDRO E	08°27'36.5"N	78°51'08"W	01-13/08/2004-01/05/2005	4.2
31	ESPIRITU SANTO	8°25'46.44"N	78°51'05.46''W	13/08/2004-01/05/2005	3.0
45	SAN JOSE	8°15'24"N	79°08'02''W	14/05/2005	3.6
53	P. GONZALES	8°24'28.1"N	79°05'02.1''W	01/05/2005	3.6
55	P. GONZALES	08°25'29"N	79°06'36"W	12-15/08/2004	5.4

From the same four stations information was also collected related to the following ecological aspects:

2b) Growth rate of the vermetids in each station for the period of August 2004 to May 2005 was determined by monitoring the difference in size from August 2004 when 10 shells were tagged *in situ* and measured with a caliper. In order to mark the 10 shells in each station, orange tags were used to mark the tip of each selected tube. Then the distance from the tag to the end of the tube was measured. Once under the water and in order to be able to find the tubes again a 50cm rope with an orange tag was knotted to each tube (or near it) so the rope would float in the water and would be easier to relocate each tube (Figure 6). To find the specific area of each reef (station) in which the tubes had been marked a triangulation method (3 fixed points in the horizon were selected so the station could be relocated under the cross section 3 bearings) was used on top of GPS. The second shell length measure was taken again in May 2005 from the tag position to the shell growth tip, so that the difference between the two measures was the growth over the time period. In order to be able to grow within the coral framework the vermetid growth rate was originally estimated to be similar to the longitudinal growth of the *Pocillopora* coral framework within which it grows. The growth rate information was useful to confirm the analysis of growth bands in the shells.

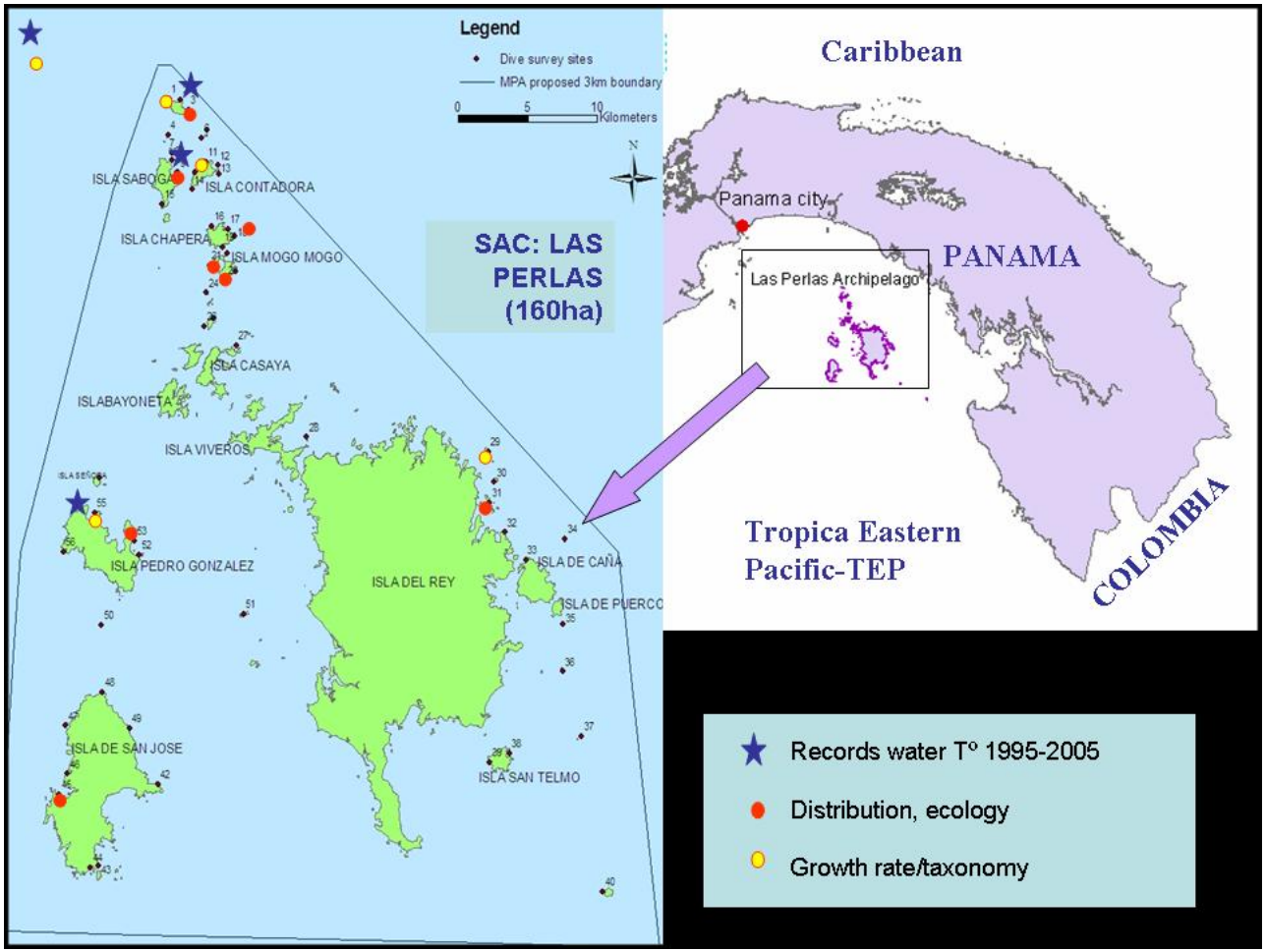

Figure 5. The Las Perlas Archipelago in the Pacific of Panama. Yellow spots are stations where samples and primary ecological information was collected (Stations 1, 12, 29, 55). Red spots are stations where only primary ecological information was collected (Stations 3, 9, 18, 21, 23, 31, 45, 53). Blue stars are stations with secondary information (database of SST for Pacheca, Saboga, Señorita and STRI). The other numbers (stations) are part of the general Darwin Project STRI-HW. Modified from GIS-Darwin Project STRI-HW University (2006) and http://www.reef.org/data/database.htm.

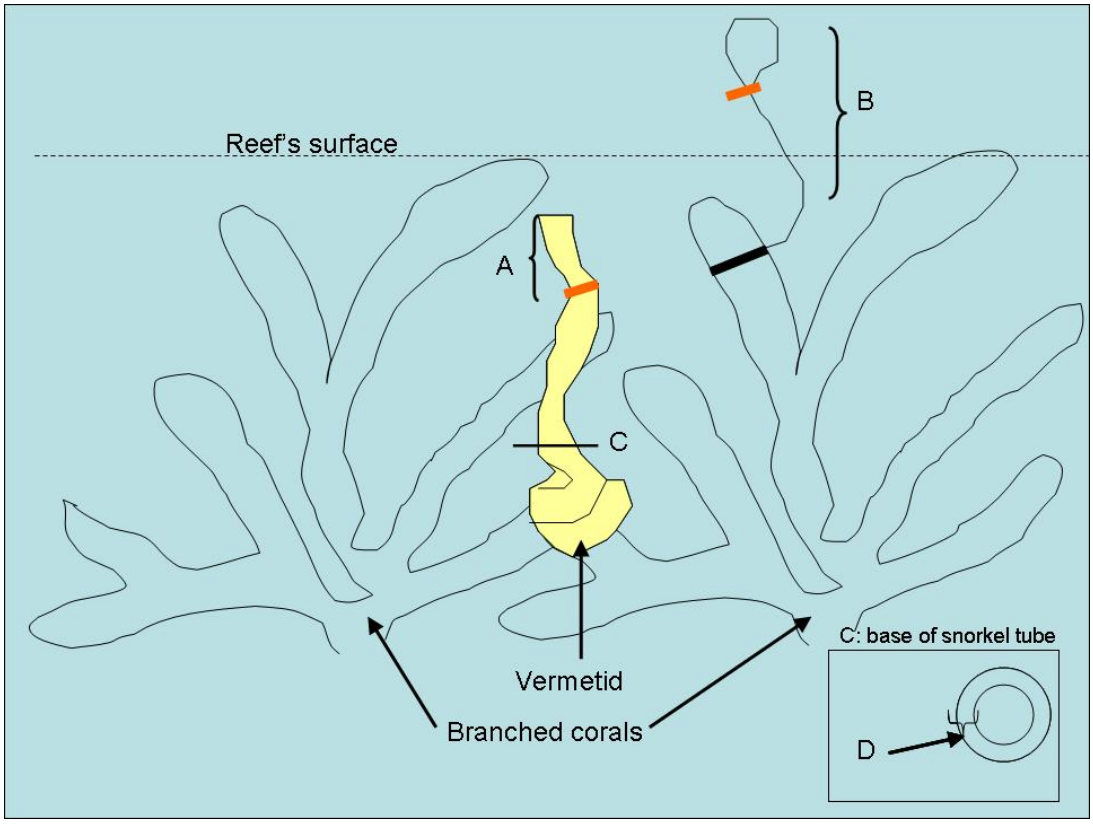

Figure 6. Diagram that explains the way in which the shells were tagged and measured *in situ*. A: distance from tag to the end of the tube that was measured in August 2004 and again in May 2005; the difference among the measures gave the growth rate during the 9 month period. B: rope of 50 cm knotted near the tube used to relocate the tube for the second measure. C: area in the base of the snorkel where a cross section was cut for growth band analysis. D: cross section from the snorkel where SEM analysis were performed to identify annual bands. In the diagram the animal is cryptic with respect to the reef surface because is under this level.

2c) Vermetid microhabitat (depth in meters, coral species associated, coral density, reef slope, mollusc level with respect to the reef surface: cryptic, exposed). These measures were taken once the tubes were selected and marked for the first time. The vermetid microhabitat was defined as the areas surrounding the tube in a radius of 50 cm. Coral density was defined as the number of *Pocillopora* branches counted in a line of 20 cm. The slope of the reef was visually estimated as the angle between a horizontal line and the reef surface.

2d) Reef complexity, or ability of the reef to support or bring ecological space to other species to live within it. This variable was evaluated by three different components:

- Reef thickness (depth of coral framework). To access the depth of the frame a measured tube (in cm) was inserted in the frame until it reached the rock or sand bottom and the depth was read directly from the tube.
- Reef density, or number of *Pocillopora* branches counted in a line of 20 cm.
- Vermetid abundance or number of vermetids in an area of 1m x 1m.

These three variables were evaluated by using 3 transects perpendicular to the coast to cover the depth range. In each transect four points were selected for measurements of the three variables for a total of 12 measuring points per reef or station.

2e) Current reef structure (the actual percent cover of each of the main reef components: total live coral and by species, total dead coral, sand, rubble, hard bottoms, algae, sponges, and main fish species). This variable was evaluated in a visual way. In this case, instead of a physical frame or quadrat, a fixed observational time-period was used to mark the borders of the station. The diver was positioned on top of the reef to select an estimated area of 5m x 5m where to carry out the visual evaluation. Within the area and in a period of maximum 30 minutes the diver evaluated the composition of the area and estimated the percentage of cover of each main component beginning from the general features such as sand vs. hard bottoms, then the hard bottom was divided into the main groups (live coral, dead coral, rock, sponges, algaes) and finally details about percentage cover of main coral and number of fish species. Additional information related to this component was also collected from other 8 stations (see red stations, Figure 5), for a total of 12 stations in the Las Perlas Archipelago.

2f) Vermetid distribution patterns, which were obtained, together with the sessile mollusc species associated (associated fauna), using quadrats of 1m x 1m (6 quadrats/station, except station 55 where 9 quadrats were measured). In this case, and once the diver had selected the area for all measurements, the diver counted and established the position of all the vermetids and any other sessile molluscs within the quadrat. Same type of measures were collected from other 8 stations (Stations 3, 9, 18, 21, 23, 31, 45, 53; see red stations in Figure 5) for a total of 12 stations (including st 1, 12, 29, 55) and 75 quadrats or fields. Each quadrat of 1m x 1m was divided into 25 sections of 20X20cm. In each section of quadrat the diver counted the number of vermetids. Therefore there was a total of 1875 counts in all the stations.

Historical secondary information about coral reef environments (e.g. percent cover of each of the main reef components) collected by scientific staff at STRI since 1992 (see Terrestrial-Environmental Sciences Program and CARICOMP of the Smithsonian Tropical Research Institute-STRI at www.stri.org) was used for comparison with the information collected during the field trips.

3.4.2. Laboratory analysis:

Taxonomy (1) and growth band patterns (2a):

With respect to the taxonomy component, the organisms were identified by traditional taxonomy using the keys of Keen (1971) and Keen and Coan (1974). Dr. Rüdiger Bieler (expert for the family Sabellaridae worldwide) suggested that complementary molecular analysis should be carried out to confirm the name of the species. The analysis was performed at the Museum of Chicago (USA) by Dr. Timothy Rawlings, who is an expert in molecular analysis for the group and is doing molecular systematic for the whole group with Dr. Rüdiger Bieler (see Rawlings *et al*. 2001). In addition to the typical *E. tulipus* with internal (brown operculum) and external form (tubes with snorkel in reef areas and without snorkel in rocky areas), other similar molluscs were identified and were catalogued as *E. tulipus* sp2 to sp6 (Annex 1). Samples of body tissue from each morphotype were collected and preserved (in 99% alcohol) for molecular analysis.

The final aim of the growth band analysis, according to the methodology of Linsley *et al.* (2000), was to identify non-typical bands (those bands with growth during perturbations such as El Niño-La Niña), and typical bands (those ones that grow during "normal" years) that could be used for calibration against records of water temperature collected over the last 10 years in Las Perlas Archipelago (see chapter 9).

Although preliminary growth band analysis, in which X-rays were used, showed that there was a possible pattern of bands in the shells, the actual X-rays were not clear due to the large amount of epifauna associated with the external parts of the shells (Figure 7). Therefore, in order to obtain much better resolution, and to avoid the noise caused by epifauna, further analysis using a modification of the methodology of Guzmán and Jarvis (1996) helped to identify the growth bands from the internal part of the shell (by longitudinally cutting the shell and taking the x-ray from inside). However, the internal X-rays did not show any good patterns either because the epifauna often had eroded from outside to even penetrate part of the internal layers and the X-rays had a low penetration in the dense material, as has been seen in other species of molluscs (Aharon 1991). Thus it was essential to select shells clean of epifauna or bio-erosion, from those collected in May 2005 and August 2004, to be sure that there was a way to detect the bands and to establish a new methodology to identify the growth bands in the shells.

A combination of stereoscope, transmitted light microscopy and the results of the annual growth rates from the marked shell was used. Each selected shell was analyzed through the stereoscope to identify potential major or monthly bands, assuming that the latest band was formed during the month in which the shells were collected (May 2005, August 2004). Then, using the microscope, minor bands between the monthly bands were identified. The information about *in situ* growth rate for the period 2004-2005, collected in Panama during the field trips, was essential at this point to produce a more accurate growth band analysis. In this case, the total length of the shell (e.g 300 mm) was divided by the average annual growth rate (e.g. 24mm) to estimate the age of the organisms (e.g. 300/24=12.5 years). Then the shell was divided in equal annual sections to see if the banding pattern for each year (length in cm occupied by the major bands each year) was repeatable and so confirm the presence of monthly bands. The final results were presented as a model for the type of growth shell in this species.

Scanning Electronic Microscopy-SEM (Philips XL30CP with PGT Spirit X-ray analysis) was used to identify potential annual bands from transverse sections of the shells and to take pictures of the bands for subsequent analyses. In this case, a cross section from the oldest part of the shell (a ring from the snorkel tube near the base of the shell; see Figure 6) was cut and prepared for the analysis. The only sample preparation required was the production of a flat surface, which was obtained by the inclusion of the samples (10 samples: PAN-04-55-8, PAN-04-55-8-A/B, PAN-04-55-4-1B, PAN-04-55-5-1B, PAN-03-55-1-A/B, PAN-03-55-4-A/B, PAN-04-55-7) in non-reactive resin and subsequent ultra-polishing (www.geos.ed.ac.uk/facilities/sampleprep). The sample had to be less than 2.5cm in diameter and less than 1.25cm thick. The final picture was presented with the scale in µm. Then, to confirm that the observed bands were indeed annual bands, the thickness of each transverse section (e.g 1500 µm) was divided by the number of years, already obtained for the monthly bands (e.g. 12.5; see calculations to estimate the age of the shells in previous parragraph), to estimate the potential thickness of each year in the section (1500 µm/12.5=120 µm each year). The thickness of each year and the number of years would be a guide to compare with the number of potential annual bands observed in the transverse section.

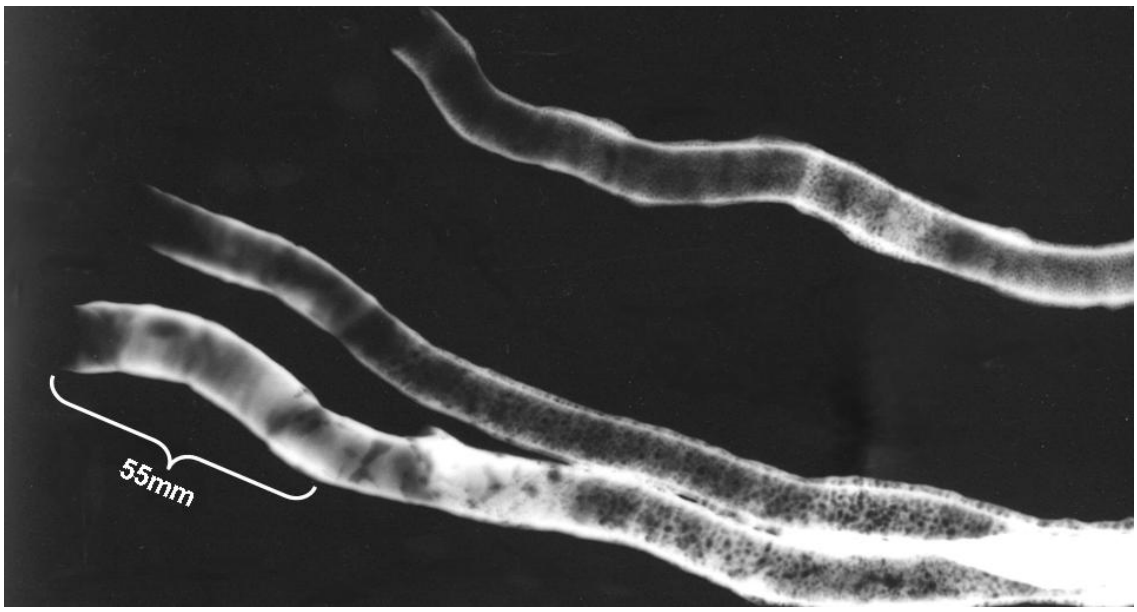

Figure 7. X-ray of vermetid shells (August 2004) collected from Las Perlas Archipelago, Panama in 2003. The scale (55 mm) refers to the top of one of the shells, without epifauna, where there is a possible pattern of growth bands. Internal x-ray analysis (no presented in this figure) showed the same pattern: some bands in areas where there was no epifauna or bio-erosion and no evident pattern in areas with epifauna or bio-erosion.

3.4.3. Statistical analysis:

Information collected during the field trips from the 12 stations and related to ecological aspects of the species was organized in an EXCEL database for the subsequent statistical analysis.

Growth band patterns (2a) and annual growth rates (2b):

The growth rate for each organism (10 per station, 4 stations in total) was obtained as the difference between the lengths collected on August 2004 and May 2005. From the 40 values obtained the average growth rate in 9 months was calculated. From these values the average annual growth rate (± standard deviation) during the period 2004-2005 were estimated and presented in graphics as average (± SD) per station and for the whole area. In the same way, the final results for the vermetids' growth band patterns in the area during the originally estimated period of 10-30 years were presented as averages (± SD). Following on from these results one typical shell was selected (tube PAN-04-55-8) for the other ecological analyses, trace elements and isotopes (see chapter 9).

To identify the most important variables responsible for the typical Vermetids microhabitat (2c), reef complexity (2d), and current reef structure (2e) PCA were performed, using the information collected during the field trips (see pages 48 and 49).

The ecological distribution pattern for the vermetids (2f) in the reef environments of Las Perlas was obtained by using the Poisson distribution of the software Minitab (Wardlaw 2001). The value represents the probability or frequency of finding squares with χ counts, χ being the occupancy number. The average count per square is M and $\chi!$ is the product of all the numbers between χ and 1 multiplied together as follows (Wardlaw 2001):

$P\ \chi = e\text{-}M * M\ \chi / \chi!$

To confirm the significance of the Poison distribution a chi-square test was performed. The hypothesis to be tested with this analysis was whether the amount of variation was within the limits of random-sampling fluctuations or whether the differences between fields (quadrats) were real (to show if the distribution of the organisms that were counted was random, uniform or aggregated).

A value of "1" in the chi-square test means a random distribution. Values greater than 1 (>1) represent aggregation, and values smaller than 1 (<1) represent uniform distribution. The formula for chi-square is:

$\chi^2 = \Sigma\ (O\text{-}E)^2/E$

Where O is the observed counts per field (quadrat) and E is the expected counts (expected if there was no variation beyond sampling fluctuations). The expected count is the arithmetic average of the observed counts in all the fields or quadrats (6 quadrats per station and 9 quadrats in station 55, total stations=12, total quadrats= 75). To obtain the value of chi-square for a probability at 5% it is essential to obtain the degrees of freedom-DF. DF is calculated as:

DF=N-1, where N represent the number of fields or quadrats analyzed (75 in this case).

3.5. Results:

3.5.1. Identification of Eualetes tulipus (Chenu 1843, ex Rouseau) according to Keen (1971, 1982):

Synonyms: *Vermetus angulatus*, *V. panamensis* Chenu 1844, ex Rousseau, MS; *V. effusus* Chenu 1844, ex Valenciennes, MS.

The species description is in agreement with those of Keen (1971, 1982). The species presented spired shells with several whorls, a round aperture, and a multispiral horny operculum. The shell was firmly attached to substrate throughout life, but differing from the original description, the shells collected from coral reef areas in Las Perlas Archipelago had the last whorl lax as a snorkel (Figure 8a). The colour of the shell was the same as the original description (ivory white to light brown) and the surface of whorls was nearly smooth. The nuclear whorls were small as in the original description by Keen (1971, 1982), with usually two to four basic whorls before the snorkel. The operculum was brown, horny, thin as a lamina (1mm thick), smaller than the aperture (diameter operculum: 5mm, average diameter of aperture: 8.8mm), left handed spiral, with four turns, concave (Figure 8b). The diameters of the aperture ranged between 5.7 and 11.4mm (average 8.8mm in the collected specimens), a little smaller than the original description (16mm for large specimens). However the length was greater (maximum length 300mm, average 100mm) than the original description (60mm). As in the original description the specimens tended to be solitary rather than colonial.

X-ray Diffraction Analysis showed that aragonite was the main component of the internal and external part of the shells, with a very low percent of calcite (only one peak of calcite with 0.01%, Figure 9). It is the mantle of the animal which is responsible for depositing the shelly matter, while the mantle margin carries the colour-depositing cells (Cernohorsky 1967). The inner layers of the shell consist of calcium carbonate (aragonite in this case). The outermost calcareous layer is generally prismatic and the mineral is deposited as vertical crystals (Rupert and Barnes 1994), of aragonite in this case. The inner calcareous layers (two or more) are laid down as aragonite sheets, or lamellae, over a thin organic matrix (Rupert and Barnes 1994). Further electronic microscope analysis (SEM) showed that in this case the mineral is deposited as vertical and very thin (<10μm) crystals of aragonite.

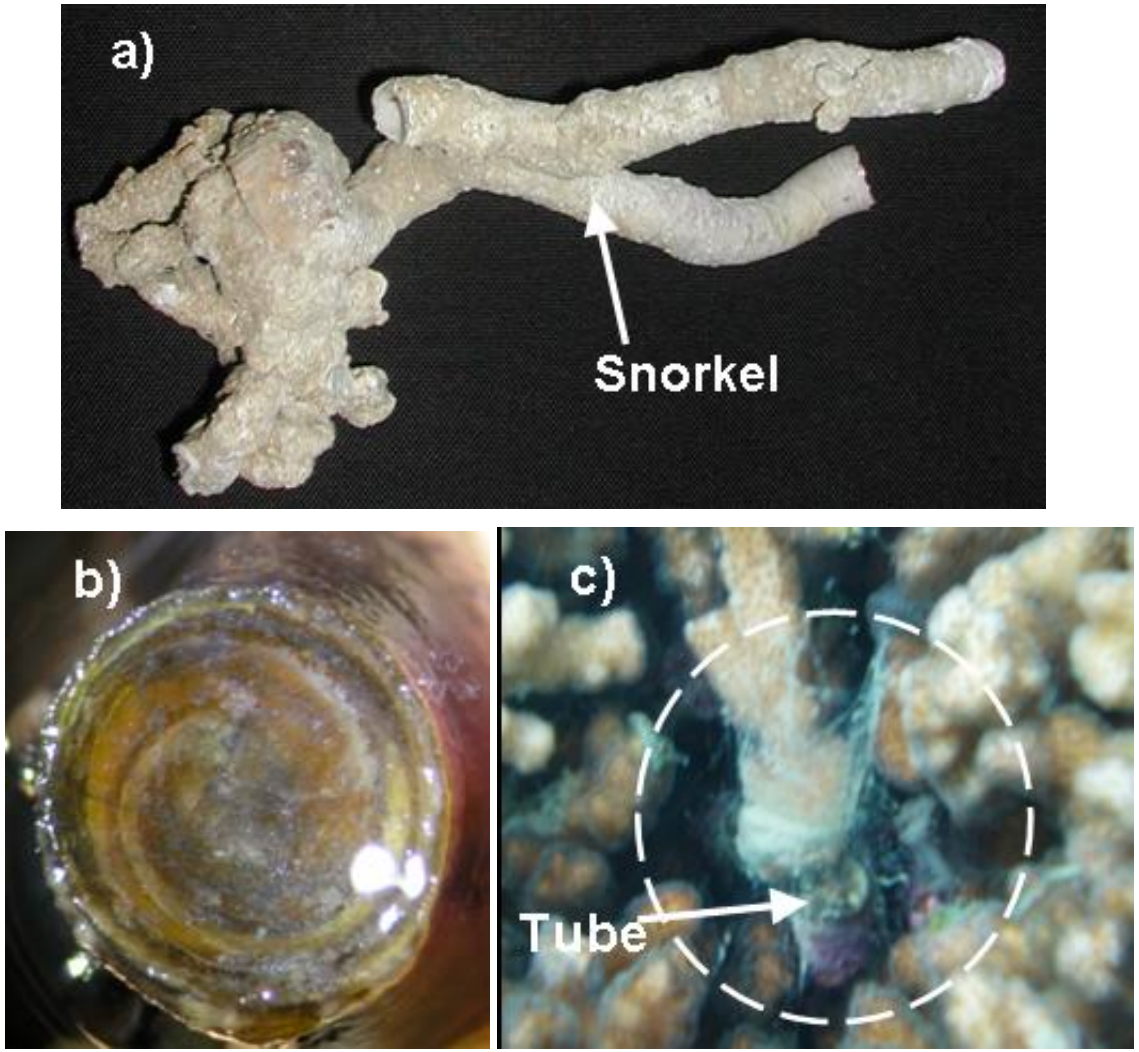

Figure 8. *Eualetes tulipus* from coral reefs in Las Perlas Archipelago. a) Shell with typical snorkel. b) Detail of operculum. c) Detail of organism in the environment feeding by re-absorption of mucus (deposit and suspension feeder by filtration). Circle shows area with mucus around the tube.

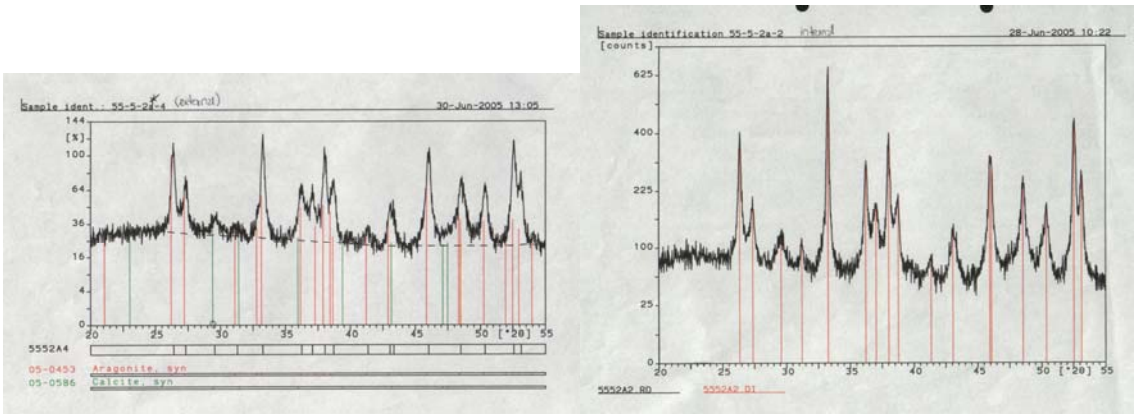

Figure 9. Example for results of X-Ray Diffraction Analysis-XRD for samples from external (left) and internal (right) part of shells from station 55 (shell PAN-04-55-5-2). Red lines are peaks of aragonite and green lines are peaks of calcite.

General observations from field work in Las Perlas Archipelago: Relatively large shells were found in rocky and coral reef areas (coral *Pocillopora damicornis*) between 2-7m depth. In the rocky areas the shell was completely coiled (nuclear whorls all the way to the top). In coral reef areas they were attached to the substratum (usually *Pocillopora* spp), with nuclear whorls relatively small and usually 4 post-nuclear whorls that are tightly coiled and attached to the substratum, then there are lax coils showing a straight tube or snorkel (Figure8a). Since they compete for space with corals, the snorkel needs to grow continuously to keep at the same level of the reef and in this way to obtain food constantly. The maximum tube length found was 300mm. Within the last post-nuclear whorl and the snorkel there are septa that mark the lowest limit of the tube that the animal uses during the adult stage. Although the original colour of the shell is white to light brown the final colour will depend on the epifauna associated with the shell. It was observed that smaller tubes (younger) tend to be brown (presence of periostracum), while the bigger ones are whiter due to the epifauna and the loss of periostracum. The vermetid appears to have an important ecological role within the reef serving on one hand as predators on small invertebrates, and on the other as food for fish and large invertebrates, as well as substratum for other sessile organisms like bryozoans, polychaetes, other molluscs, and even corals.

Nutrition: The species *Eualetes tulipus* appears to be a filter-feeder (deposit and suspension feeder) since a mucus bolus was often observed to be used to capture fine particles (Figure 8c).

Molecular analysis:

Amplification and sequence of 1700bp portions of 12S and 16S for samples of *E. tulipus* sp1 from Pedro Gonzáles and Chapera Islands (St 55, 18) confirmed the specimens to be *E. tulipus*. They grouped with others vermetids from Panama and the Caribbean. Analysis of '*E. tulipus* sp3' (Taboga station 101) indicated that it was really *Vermetus erectus* (a deep water vermetid). The specimens '*E. tulipus* sp1' were those used for all the ecological analyses.

3.5.2. Annual growth rate:

The results refered to are the longitudinal growth of the shell (tube). The results of the analysis showed an average annual growth rate of 24.2mm (average longitudinal growth rate in 9 months: 18.1mm; Table 4). However there were large variations amongst the samples (standard deviation for the average growth rate in 9 months: 15.6; Figure 10), including null results for moluscs that died between the first and second measure (August 2004-May 2005), especially in stations 1 (Pachequilla) and 29 (San Pedro). In Table 4 there are also results of measurements of width growth rate (annual width growth for the period 2004-2005 was estimated as 1.8mm). Due to the shape of *E. tulipus* shells, the width growth rate had much smaller values (between 0-4mm/year) in comparison with the longitudinal growth rate (values between 0-66mm/year). Therefore, the larger variations in the measurements for width growth rate are considered to be the result of the difficulty to measure the variable in the field with calipers. The above, and the fact that both variables should have a similar behaviour, were the reasons to use only the results of longitudinal growth rate for subsequent analysis.

Table 4. Values of longitudinal growth rate (and width growth rate) for each of four stations and for the whole area for the period August 2004-May 2005, including estimations of annual growth rates for the period 2004-2005. Data was collected from marked and recaptured vermetids. Growth in width was taken during the fild trips from the tip of the snorkel (two measures per tube) with a caliper. NA: no availability due to loss of the shell (or mark) for the second measure in 2005.

Station	Specimen number	LENGTH 2005(mm)	LENGTH 2004(mm)	Growth rate (2004-2005)	**average growth rate (length mm)**	WIDTH 2005(mm)	WIDTH 2004(mm)	Growth rate (2004-2005)	average growth rate (width mm)
1	1	12.4	12.5	-0.1		9.1	9.1	0	
1	2	NA	6.6	NA		NA	9.4	NA	
1	3	5.8	NA	NA		7.5	NA	NA	
1	4	12.2	13.5	-1.3		10.7	9.2	1.5	
1	5	16.5	12.4	4.1		10.7	8.7	2	
1	6	15.4	12.9	2.5		6.9	6.9	0	
1	7	8.2	7.7	0.5		7.9	6.1	1.8	
1	8	NA	3.8	NA		NA	9.6	NA	
1	9	NA	9.2	NA		NA	7.4	NA	
1	10	28.9	7.5	21.4	**7.1**	9.8	5.7	4.1	1.6
Stand. Dev.					**9.6**				1.5
12	1	35.6	4.8	30.8		9.6	9	0.6	
12	2	29,8	9.3	20.5		10	10.3	-0.3	
12	3	73	6.6	66.4		10.7	7.5	3.2	
12	4	32.7	13.3	19.4		10.6	7.4	3.2	
12	5	18.1	9.5	8.6		8.4	8.4	0	
12	6	7.9	8.9	-1		6.9	6.9	0	
12	7	53.1	5.1	48		9.2	7.6	1.6	
12	8	17	2.3	14.7		11.5	11.4	0.1	
12	9	35.4	4.4	31		10.7	7.1	3.6	
12	10	NA	19.65	NA	**29.9**	NA	7.9	NA	1.5
Stand. Dev.					**19.1**				1.6
29	1	NA	11.5	NA		NA	10	NA	
29	2	10	14.3	-4.3		10.6	10.5	0.1	
29	3	14.3	9.5	4.8		11.2	9.9	1.3	
29	4	NA	13.1	NA		NA	9.5	NA	

Station	Specimen number	LENGTH 2005(mm)	LENGTH 2004(mm)	Growth rate (2004-2005)	**average growth rate (length mm)**	WIDTH 2005(mm)	WIDTH 2004(mm)	Growth rate (2004-2005)	average growth rate (width mm)
29	5	NA	13.7	NA		NA	10.3	NA	
29	6	21.8	3.6	18.3		10.1	9.1	1	
29	7	13.2	3.8	9.4		10.5	10	0.5	
29	8	NA	5.9	NA		NA	11	NA	
29	9	34.8	16.3	18.4		10.2	9.2	1.1	
29	10	NA	5.5	NA	**12.7**	NA	10.9	NA	0.8
Stand. Dev.					**6.8**				0.5
55	1	19.1	4	15.1		NA	NA	NA	
55	2	10	4	6		NA	NA	NA	
55	3	37	22	15		NA	NA	NA	
55	4	25	13	12		NA	NA	NA	
55	5	17.9	4	13.9		NA	NA	NA	
55	6	NA	7	NA		NA	NA	NA	
55	7	NA	17	NA		NA	NA	NA	
55	8	NA	11	NA		NA	NA	NA	
55	9	NA	16	NA		NA	NA	NA	
55	10	NA	6	NA	**12.4**	NA	NA	NA	NA
Stand. Dev.					**3.8**				NA
Longitudinal growth rate in 9 months					**18.1**			Width **growth rate in 9 months**	1.4
Longitudinal growth rate 2004-2005					**24.2**			Width **growth rate** 2004-2005	1.8
Standard dev for values in 9 months					**15.6**			**Standard dev for values in 9 months**	1.3

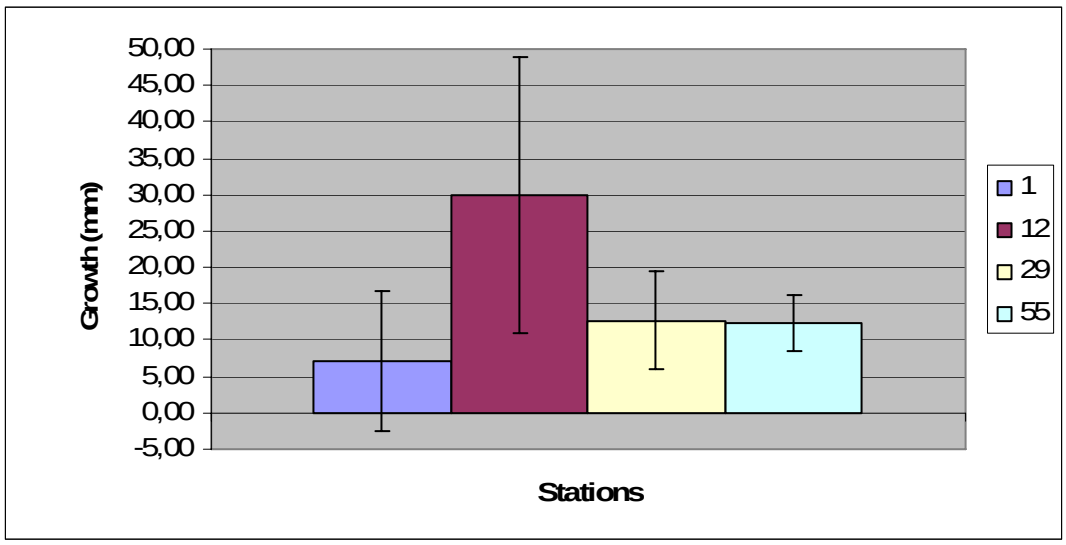

Figure 10. Longitudinal growth rate (± SD) of shells from four stations during 9 months: August 2004-May 2005. For station locations see figure 5.

3.5.3. Growth band patterns:

Observations with stereoscope of several shells from Panama (mainly St. 55) show that there are detectable monthly (1 pair of spring tide bands) and daily bands (Figure 11a). Counting back from the day in which the organisms and shells were collected there were evident brown bands along the shell (Figure 11b). It was also evident that there was more space between some of those dark bands, creating groups of spaced bands and compacted bands (see model in Figure 11a). Then, counting back from the last band that was created before the shells were collected, and based on the assumption that the last band in tip of each shell corresponded to the band of the month in which the shell was collected (in this case August 2004), it was possible to identify that there were 8 spaced bands and then 10 compacted bands. At this point the results of the annual growth rate were very useful to confirm that each 24mm (approximately a year according to the average annual growth rate) correspond to a group of spaced and compacted bands. Thus the 10 compacted bands correspond to the months December to April, with a pair of bands per month (spring tide bands) and related to the upwelling period. The other 8 bands correspond to a pair of bands for the months May to August (Figure 11a). Observations of each brown band under stereoscope and microscope (5X with normal microscope or under good stereoscope) showed that each visible brown band corresponded to three or four minor brown bands and between each group of brown bands there were between twenty and twenty-four white bands. Therefore, these minor bands correspond most probably to daily bands and the group of three to four minor brown bands corresponds to daily bands created during the spring tides (Figure 11a).

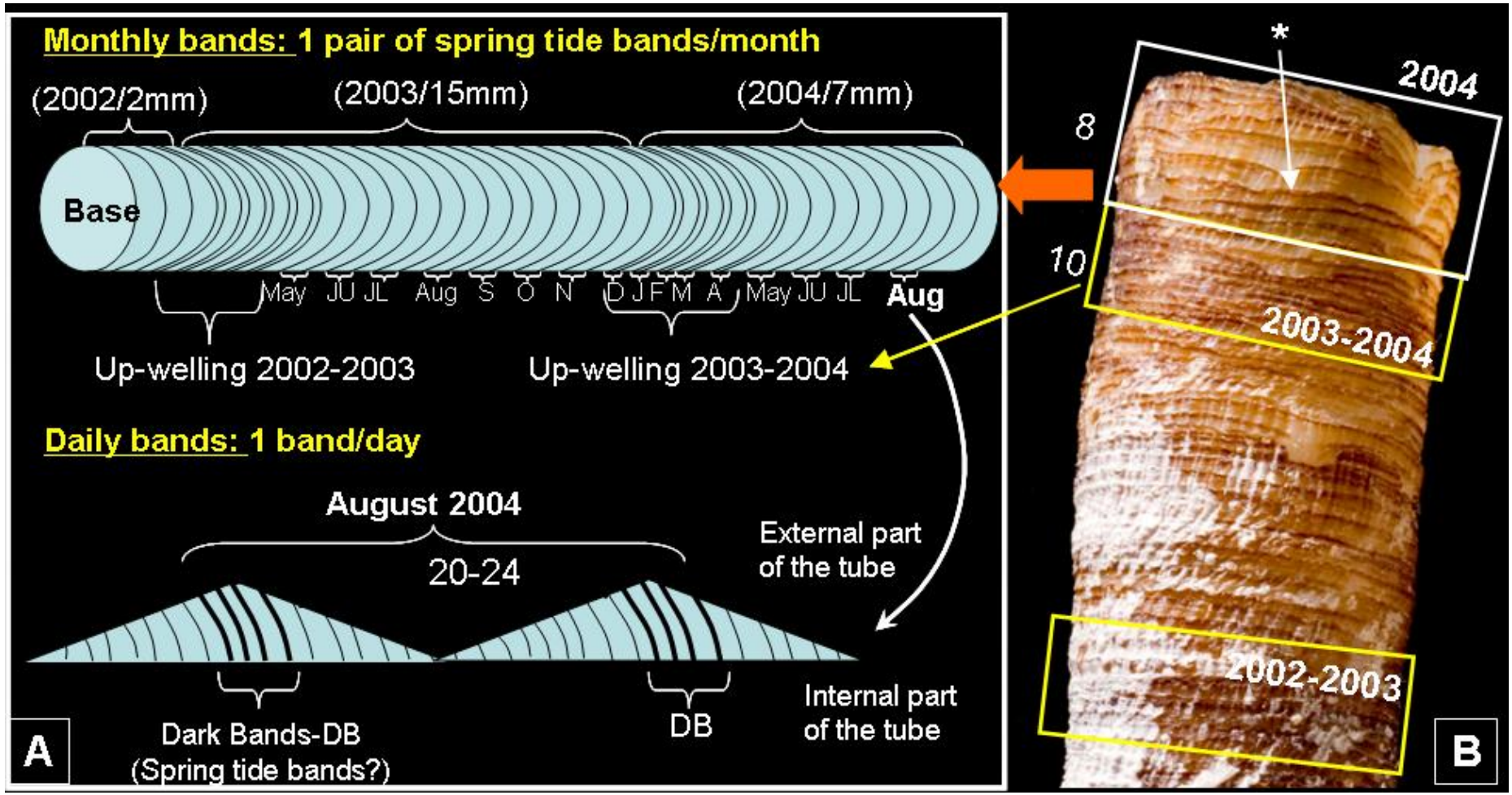

Figure 11. Monthly and daily bands in *E. tulipus*. A: Scheme of spring tide (monthly) and daily bands from sample PAN-04-55-3. B: Picture of monthly bands in PAN-04-55-9. *: spring tide band from 2004 during non upwelling period.

Observations of 10 transverse sections of *E. tulipus* shells from Panama (block samples: PAN-04-55-8, PAN-04-55-8-A, PAN-04-55-8-B, PAN-04-55-4-1B, PAN-04-55-5-1B, PAN-03-55-1-A, PAN-03-55-1-B, PAN-03-55-4-A PAN-03-55-4-B, PAN-04-55-7) with Scanning electronic microscope (SEM) showed also some banding that could be related to annual bands (Figure 12). Here once again the growth rate results were useful to confirm the assumption of annual bands. In this case the total length of the shell (e.g 320 mm for PAN-04-55-8) was divided by the average annual growth rate (24mm) to estimate the age of the organisms (e.g. 320/24=13.3 years or approximately 14 years). Then the thickness of the transverse section (e.g 1614 µm for PAN-04-55-8) was divided by the number of years (13.3) to estimate the theoretical size of annual bands in the transverse section (1614 µm/13.3=121.4 µm each year). The theoretical size of the annual bands and the number of years worked only as a guide to compare with the number of bands observed in the 10 transverse sections. Figure 12 shows the internal layers of one shell from the youngest part (bottom) to the oldest part (top). Dividing the section every time that there was a strong white line it is possible to count 14 sections, including a small section in the youngest part of the shell (bottom) that would correspond to part of 2004. The oldest part or end of the internal layers is market by a fracture and then by holes created by bio-eroders.

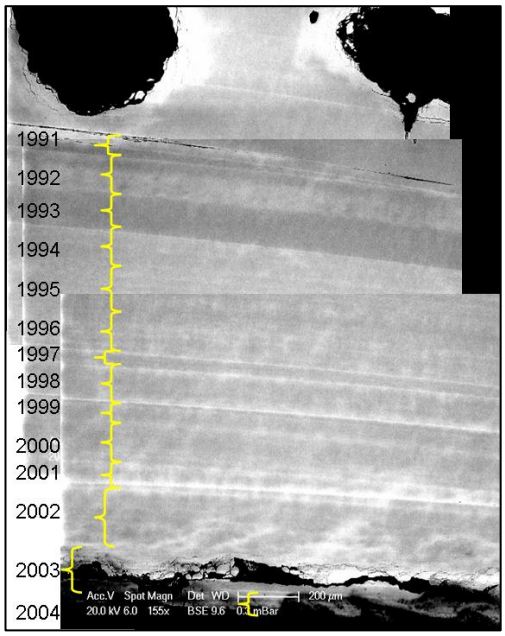

Figure 12. SEM analysis for sample PAN-04-55-8 (composite image of transverse section) in which are evident more than 13 bands and a small section that would correspond to the year in which the sample was collected, in this case 2004. Yellow brackets indicate the width of the 14 annual bands obtained by dividing the transverse section every time that there was a strong white line. The bottom of the image corresponds to the internal part of the snorkel (see Figure 6 for details of cross sections), where the youngest band (2004) was growing. Top of the image corresponds to the end of internal layers in the shell (holes are bioerosion by epifauna), where the oldest band was deposited. Total transect size:1614µm. Age of shell: 13.3 years. Theoretical size of annual bands in the transverse section:1614 µm/13.3=121.4µm

3.5.4. Microhabitat :

Observations in the field with respect to microhabitat showed that, in the branched coral reef environments of Las Perlas Archipelago, the vermetids prefer to live around 4m depth, in environments with average slope of 23º, surrounded by corals of the species *Pocillopora damicornis*, with a coral cover higher than 80% and in general of 95%, and an average density of 12.6 branches/20cm. In most of the cases the vermetid top was 1-2cm under the reef surface (cryptic). The observations showed that animals extending above the reef surface are most exposed to predation and those under 2cm of the reef surface may die as a result of overlapping or overgrowth by the corals (Table 5).

PCA results with the six variables measured for microhabitat showed that there are two main components that explain 63.9% of the microhabitat observed for the vermetid *E. tulipus* in Las Perlas Archipelago (Figure 13). The first Principal Component (PC1) has a variance (eigen value) of 2.316 and accounts for 38.6% of the total variance (contains 38.6% of the components necessary for the microhabitat). The second component (PC2) has a variance of 1.516 and contains 25.3% of the other variables (cumulative value = 0.639). The results also show that PC1 results of the combination of coral spp, slope and coral cover (Figure 13, Annex 2), because these variables have the same sign and their coefficients present the higher values in this component. Coral density is therefore the main variable in PC2 (see Annex 2). Depth presented similar values in the first two components, but not so large to be part of any of them.

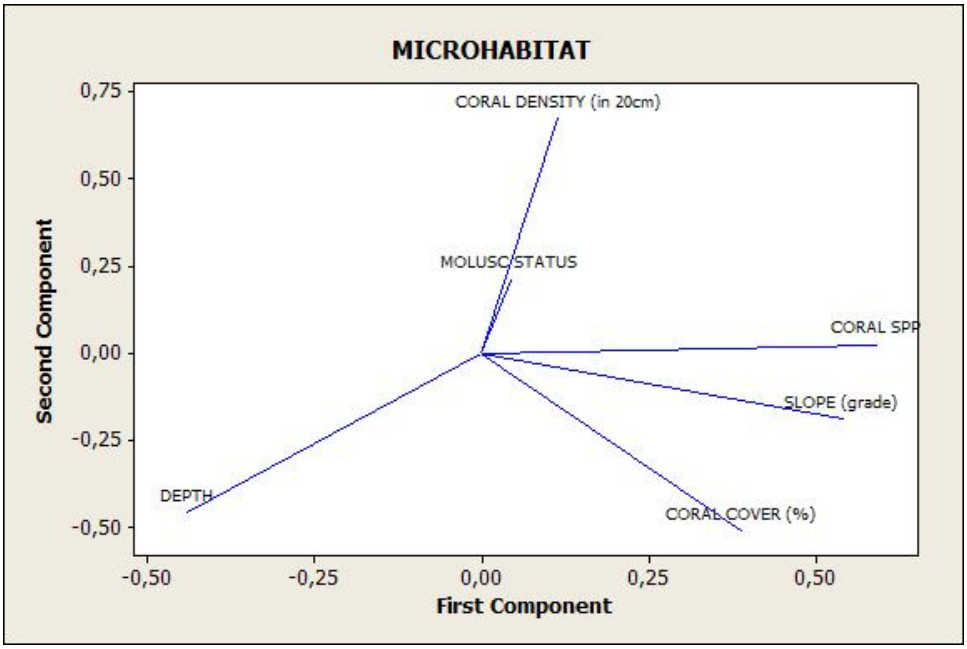

Figure 13. PCA for the microhabitat section. Results for the first two components that explain 63.9% of the microhabitat observed for the vermetid *E. tulipus* in Las Perlas Archipelago. The eigen value for PC1 (2.316) contains 38.6% of the components necessary for the microhabitat. The eigen value for PC2 (1.516) contains 25.3% of the other variables (cumulative value = 0.639). See Annex 1 for complete results of the microhabitat-PCA.

3.5.5. Reef complexity:

The reef complexity, or ability of the reef to support or bring ecological space to other species to live within, was measured with respect to 3 components: a-reef thickness (depth of coral frame), b-reef density (number of coral branches in a line of 20cm) and, c-vermetids abundance (number of vermetids in 1m x 1m).

The results (Table 6) show that the reef thickness varied between 32 and 100cm (average: 60cm), the reef density between 9 and 21 branches/20cm (average: 16 branches/20cm) and, the vermetids abundance between 0 and 11 organisms/m^2 (average: 2 organisms/m^2).

Results of PCA with the three variables measured showed that there are two main components that explain 79.1% of the requirements of *E. tulipus* for settlement success and survival in a coral reef, or requirements in terms of complexity of the reef (Figure 14). The first Principal Component (PC1) has variance (eigen value) of 1.370 and accounts for 45.8% of the total variance (contains 45.8% of the requirements for settlement). PC2 has variance of 1.003 and contains the other 33.4% of the requirements for settlement (cumulative value = 0.791). The results also show that the main variable responsible for the results in PC1, or with the higher coefficient, is the density of coral branches (Figure 14, Annex 3). PC2 is mainly explained or represented by the thickness of the reef. The abundance of vermetids presents a minor proportion in the first two components, although the graphic results (Figure 14) shows that it is more related with PC1.

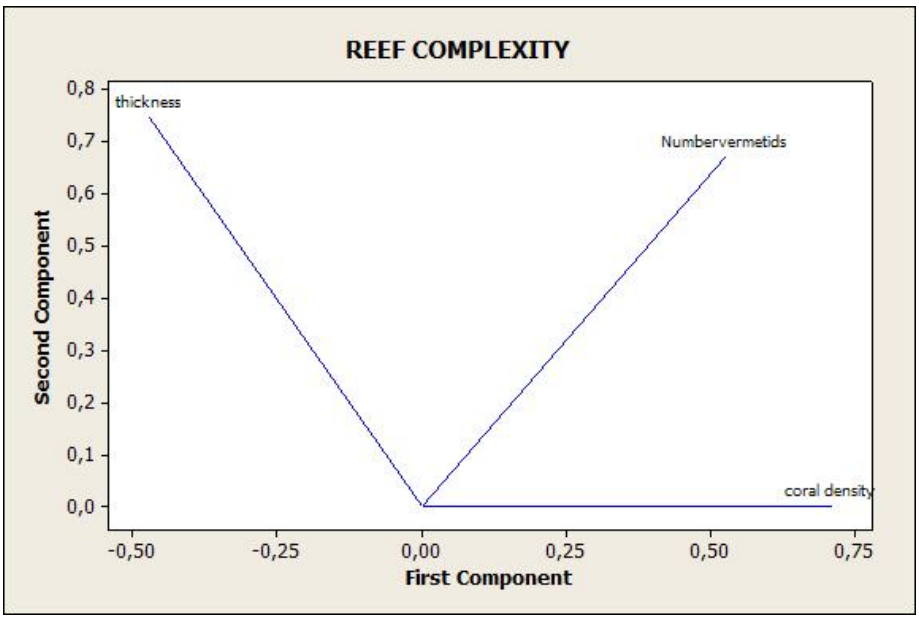

Figure 14. PCA with respect to reef complexity component. Results for the first two components that explain 79.1% of the requirements of *E. tulipus* with respect to reef complexity in Las Perlas Archipelago. Eigen value for PC1 (1.370) explains 45.7% of the variability observed. Eigen value for PC2 (1.003) explains other 33.4% of the variability (cumulative value = 0.791). See Annex 2 for complete results of the reef complexity-PCA.

3.5.6. Current reef structure:

Table 7 shows the variables measured with respect to the current reef structure. In general the coral reef environment where the vermetids were found had a live coral cover higher than 60%, with stations in which the live coral cover was even near 100% and, therefore, the other components (sponges, macroalgae, octocorals, even dead corals) were very low. The main coral species were the branched corals of the genera *Pocillopora*, which create a very thick reef surface with very small undulations (general topography 2).

PCA results with the seven variables measured (same variables as in Table 7 plus number or fish species, Annex 4) showed that there are three main components that explain 71.7% of the structure of the coral reefs in which there were vermetids (Figure 15). PC1 has a variance (eigen value) of 2.348 and accounts for 33.5% of the total variance (explains the 33.5% of the observed structure). PC2 has a variance of 1.664 and explains the other 23.8% of the observed structure. PC3 has variance of 1.005 and explains another 14.4% of the actual structure (cumulative value = 0.717). PC1 seems to be mainly represented by live coral cover because it is variable with the highest coefficient (Figure 15, Annex 4). In the same way, PC2 is mainly represented by dead coral cover and PC3 is mainly represented by the number of coral species. Although the number of coral species, depth and the number of fish species presented the same sign as live coral cover in PC1, their values were much lower (Figure 15) and therefore of minor significance. The number of fish species became important in the fourth component (Annex 4). Due to the fact that Figure 15 is a graphic expression of the variables in two dimensions it can only explain the first two components. A graphic in three dimensions would show the coral species in the third dimension.

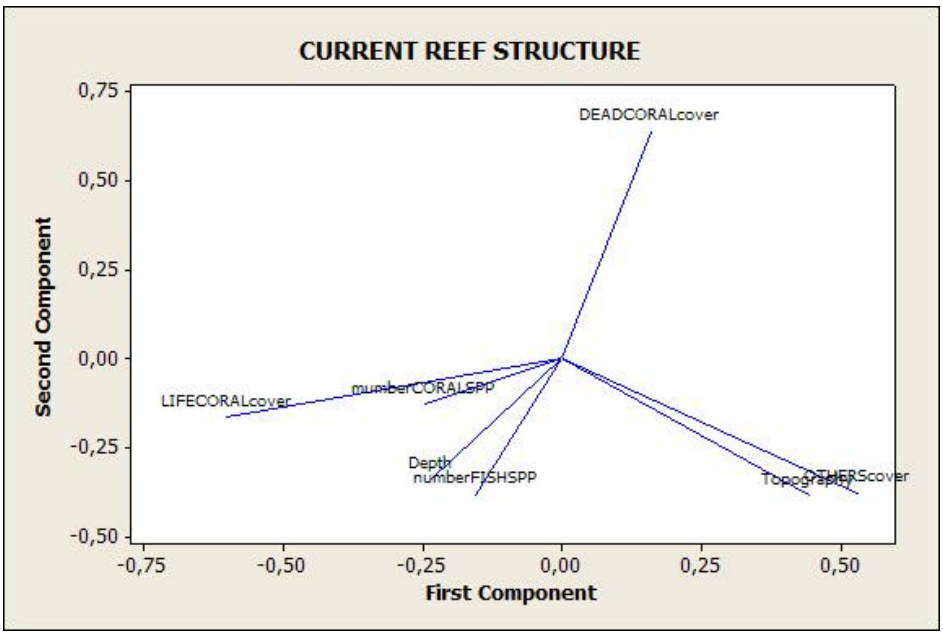

Figure 15. PCA for the current reef structure section. Results for the first two components that explain 57.3% of the actual structure for the coral reefs in Las Perlas Archipelago. Eigen value for PC1 (2.348) explains 33.5% of the variability observed. Eigen value for PC2 (1.664) explains the other 23.8% of the variability (cumulative value = 0.573). See Annex 3 for complete results of the current reef structure-PCA.

3.5.7. Distribution pattern of vermetids:

A total of 75 quadrats and 1875 counts (25 counts per quadrat) were done at the 12 stations (see Figure 5) selected for this analysis. The results of the total counts/quadrat showed values between 0 (some quadrats in Pedro Gonzales, Pedro Gonzales East, San Pedro, Saboga, Mogo Mogo, Espitirtu Santo and, Pacheca islands) and 9 organisms/quadrat (station 12, quadrat 4, Contadora Island). A first approach to this information (Figure 16) showed that "0" was the most common value, even with a selection of those stations where there were vermetids present (Figure 16 bottom). This would presume a confirmation for distribution in aggregates, which was the assumption from observations in the field. However, the Poisson frequency (Table 8) and posterior chi-square test (Table 9) proved a statistical random distribution (see value chi-square probability=1 in Table 9).

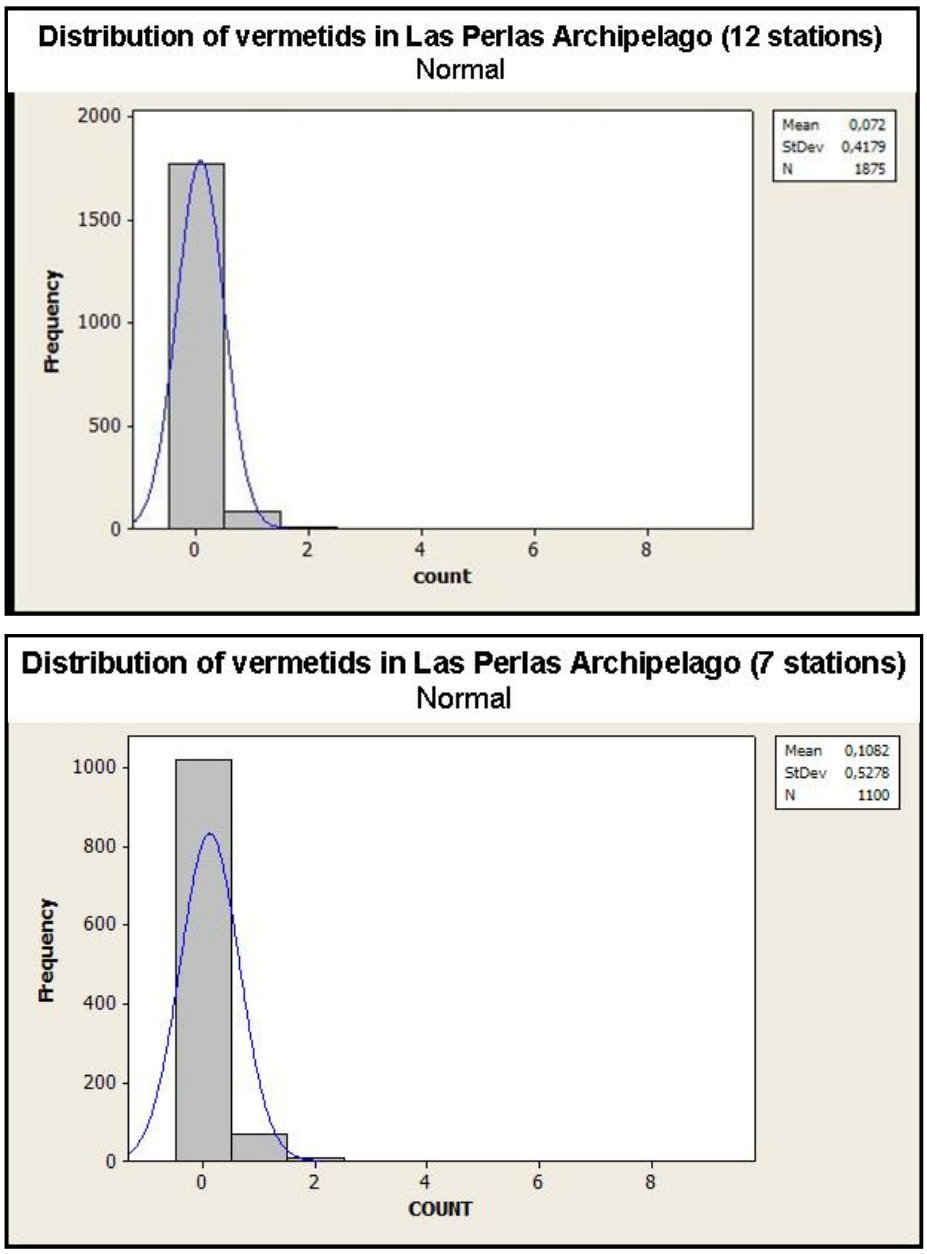

Figure 16. Histogram of frequency of counts of vermetids in Las Perlas Archipelago. Top shows the results for all the stations (see st 1, 3, 9, 12, 18, 21, 23, 29, 31, 45, 53, 55 in Figure 5. Mean=0.072, SD=0.4179, N=1875 counts). Bottom shows the results for stations where vermetids were found (see st 1, 3, 12, 29, 45, 53, 55 in Figure 5. Mean=0.1082, SD=0.5278, N=1100 counts).

With respect to the associated fauna, Table 10 shows the main groups of associated fauna in the stations and the number of species found per station and taxonomic group. The fact that Porifera and Vertebrata are the main taxonomic groups is partly a result of the methodological approach used for information gathering, the identification of the main features on the reef by visual reef cover (that allowed a reasonably rapid assessment of any component that was not coral, in this case sponges) and an extra approach focussed on visual identification of fish species.

Table 5. Observations from the field with respect to microhabitat.

Station	Location	Replicates	Depth (m)	Coral spp	Coral cover (%)	Coral density (in 20cm)	Slope gradient (degrees)	Status of vermetids in the reefs
1	Pachequilla	10	6.06	*Pocillopora. undata*	Range: 70-95 Average: 88.5	Range: 9-15 Average: 11.1	Range: 5-10 Average: 7.8	Cryptic (x7), surface (X2), semi-cryptic
9	Saboga ne	3	2.12	NA	Range: 50-85 Average: 65	Range: 14-21 Average: 16.6	Range: 0-5 Average: 3.3	Cryptic (x3)
12	Contadora	10	(2.42-5.15) Average: 3.06	*Pocillopora damicornis*	100	12.5	Range: 40-45 Average: 43.5	Cryptic (x7), surface (x3)
29	San Pedro (east)	10	(4.84-5.15) Average: 5.12	*P. damicornis*	Range: 95-100 Average: 97.5	Range: 10-14 Average: 11.7	Range: 10-20 Average: 18.3	Cryptic (x10)
55	Pedro Gonzales	10	(2.42-3.33) Average: 2.72	*P. damicornis*	Range: 80-100 Average: 95.2	Range: 9-20 Average: 12.9	15	Cryptic (x9), surface
		AVERAGE.	3.5		89.2	13.0	17.6	

Table 6. Variables related to reef complexity. Morf. 2: *E. tulipus* morphotype 2. NA: not available

Station	Location	Transect Nº	Nº of measurements	Depth of reef (m)	Reef thickness (cm)	Nº vermetids/m^2	Nº of coral branches/20 cm
3	Pacheca east	1	4	Range: 3.9-4.5 Average; 4.2	Range: 32-59 Average: 46	0/3 (2 of morf.2)/ 2 (morf. 2)/1 (morf.2)	Range: 12-20 Average: 16
3	Pacheca east	2	4	Range: 5.2-5.4 Average: 5.35	Range: 45-85 Average: 71	1 (empty tube)/1/ 0/0	Range: 13-19 Average: 16
3	Pacheca east	3	4	Range: 4.8-5.4 Average: 5.05	Range: 48-70 Average: 58	1 (morf. 2)/2 (morf. 2)/ 2 (empty tubes)/0	Range: 15-17 Average: 16
55	Pedro Gonzales	1	8	Range: 2.7-4.2 Average: 3.52	Range: 40-80 Average: 52	2 (morf. 2)/6 (5 of morf. 2)/ 0/3 (2 empty tubes)/ 0/2/ 3 (1 empty tube/1 morf. 2)/ 1 (morf. 2)	Range: 14-21 Average: 17
55	Pedro Gonzales	2	8	Range: 3-4.5) Average: 3.52	Range: 20-70 Average: 48	2 (1 empty tube)/1 (morf. 2)/ 0/9 (4 of morf. 2)/0/ 11 (3 of morf. 2)/5/0	Range: 16-22 Average: 18
55	Pedro Gonzales	3	3	Range: 3.3-4.5 Average: 3.8	Range: 40-65 Average: 55	5 (2 of morf. 2)/ 4 (3 of morf. 2)/2	Range: 18-19 Average: 18.6
12	contadora	1	6	Range: 3-3 Average:3	Range: 50-100 Average: 71	0/5/2/1/5 (morf. 2)/1	Range: 11-20 Average: 15
12	contadora	2	4	Range: 2.4-3.3 Average: 2.7	Range: 55-76 Average: 64	4 (2 of morf. 2)/0/0/ 6 (2 of morf. 2)	Range: 12-15 Average: 13.3
12	contadora	3	6	Range: 2.7-4.2 Average: 3.3	Range: 35-90 Average: 72	2/0/3 (morf. 2)/ 0/0/0	Range: 10-16 Average: 12.3
			AVERAGE	3.8	60	2.1	15.7

Table 7. Main variables related to the current coral reef structure. Topography: complexity of the bottom. 1= no vertical faces or very low structure, 2= low to slight undulations (0-0.5m), 3= moderate structure with outcrops, boulders, crevices and overhangs (0.5-1m), 4 = high structure with outcrops, many overhangs, crevices and boulders (1.5->2m). OTHERS: is related to other benthic components such as macroalgae, crustrose coralline algae, octocorals, or sponges. Information from August 2003, May 2004 and August 2005 was obtained from STRI and the program CARICOMP.

St.	Date	Topography*	Depth (m)	Coral species	live coral (%)	dead coral (%)	others (%)	total (%)
1	01/05/2004	2	5.8	*Pavona gigantea, Pocillopora gigantea, P. damicornis, P. undata*	91.6	0.0	8.4	100.0
1	17/05/2005	2	7.3	*Pocillopora capitata, P. elegans, P. damicornis, Pavona gigantea*	90	9	1	100.0
3	18/05/2005	2	5.4	*P. capitata, P. damicornis, Psamocora* sp.	70	29	1	100.0
9	01/08/2003	2	2.4	*Psamocora* sp, *Pavona clavus, Pavona gigantea, P. damicornis, P. gigantea, Porites panamensis, Psamocora obtusa, Psamocora stellata*	60.2	0.2	39.6	100.0
9	18/05/2005	2	4.5	*P. damicornis, P. elegans, Pocillopra damicornis, P. inflata, P. capitata, Psamocora* sp, *Gardineroselis planulata*	78	21	1	100.0
12	01/05/2004	2	3	*P. gigantea, Porites lobata, Psamocora stellata*	79.6	0.5	19.9	100.0
12	11/05/2005	2	6.7	*P. damicornis, P. gigantea, P. elegans, P. lobata, Pavona gigantea, P. Panamensis*	75	24	1	100.0
18	18/05/2005	2	4.8	*P. capitata, P. damicornis, P. gigantea, P. elegans*	95	4	1	100.0
19	01/05/2004	2	6.4	*Pavona gigantea, P. gigantea, Psamocora stellata*	61.8	0.5	37.7	100.0
21	01/05/2004	2	3.9	*P. gigantea, Porites panamensis, Psamocora stellata*	66.0	0.0	34.0	100.0
21	17/05/2005	2	3.6	*P. capitata, P. elegans, Psamocora* sp., *Porites lobata*	90	9	1	100.0
21	17/05/2005	2	3.6	*P. capitata, P. elegans, Psamocora* sp., *Porites lobata*	50	49	1	100.0
21	17/05/2005	2	3.6	*P. capitata, P. elegans, Psamocora* sp., *Porites lobata*	70	29	1	100.0
23	12/05/2005	2	3.9	*P. capitata, P. damicornis*	95	4	1	100.0
29	01/05/2004	2	6.7	*P. gigantea, Porites lobata*	93.7	0.2	6.1	100.0
29	13/08/2005	2	4.2	*P. capitata, P. damicornis, P. gigantea, Porites lobata*	99	0	1	100.0
31	01/05/2004	2	5	*P. gigantea*	40.0	0.1	59.9	100.0
31	13/08/2005	2	3	*P. damicornis*	20	79	1	100.0
38	01/08/2004	3	4.9	NA	12.4	0.0	87.6	100.0
45	01/08/2004	2	3	*P. gigantea, Porites panamensis, Psamocora stellata*	83.0	0.0	17.0	100.0
45	14/05/2005	2	3.6	*P. damicornis, P. inflata, P. capitata*	95	4	1	100.0
53	01/05/2004	2	2.4	*Pavona gigantea, P. gigantea*	44.7	0.0	55.3	100.0
53	15/05/2005	2	3.6	*P. adamicornis, Psamocora* sp.	25	50	25	100.0
55	01/08/2003	2	5.8	*Porites panamensis, P. damicornis, P. gigantea*	96.4	0.0	3.6	100.0
55	12/08/2004	2	5	*P. damicornis, P. inflata, P. capitata, P. elegans, Porites lobata, Pavona gigantea*	99	0	1	100.0

Table 8. Poisson frequencies and chi-square test for all stations (A: 12 stations) and those with vermetids (B: 7 stations). Ocupancy number: range of values or categories observed for the counts in each quadrat. Observed counts: Number of times that each category was observed.

A	**Occupancy number**	**Observed count**	**Poisson frequency**	**Poisson %**	**Expected count (1875)**	**O. counts-E. counts**
	0	1778	0.931	93.053	1744.8	33.25
	1	81	0.067	6.700	125.6	-44.62
	2	10	0.002	0.241	4.5	5.48
	3	1	0.000	0.000	0.1	0.89
	4	1	0.000	0.000	0.0	1.00
	5	1	0.000	0.000	0.0	1.00
	6	1	0.000	0.000	0.0	1.00
	7	1	0.000	0.000	0.0	1.00
	9	1	0.000	0.000	0.0	1.00
	TOTAL	**1875**	**1**	**100**	**1875**	**0,00**
B	**Occupancy number**	**Observed counts**	**Poisson frequency**	**Poisson %**	**Expected count (1100)**	**O. counts-E. counts**
	0	1018	0.897	89.746	987.2	30.8
	1	67	0.097	9.709	106.8	-39.8
	2	9	0.005	0.525	5.8	3.2
	3	1	0.000	0.019	0.2	0.8
	4	1	0.000	0.001	0.0	1.00
	5	1	0.000	0.000	0.0	1.00
	6	1	0.000	0.000	0.0	1.00
	7	1	0.000	0.000	0.0	1.00
	9	1	0.000	0.000	0.0	1.00
	TOTAL	**1100**	**1.000**	**100.000**	**1100.0**	**1E-04**

Table 9. Chi-square test for stations in which vermetids occurred (stations 1, 3, 12, 29, 45, 53, 55).

station/field	field observed	station/field	field observed	station/field	field observed	station/field	field observed
1.1	1	12.1	7	45.1	2	55.1	1
1.2	8	12.2	3	45.2	0	55.2	3
1.3	17	12.3	1	45.3	3	55.3	0
1.4	7	12.4	9	45.4	3	55.4	0
1.5	10	12.5	3	45.5	2	55.5	0
1.6	8	12.6	1	45.6	4	55.6	0
3.1	3	29.1	1	53.1	1	55.7	2
3.2	1	29.2	3	53.2	0	55.8	3
3.3	1	29.3	1	53.3	0	**field expected**	**2.7**
3.4	0	29.4	0	53.4	0	**chi-square calculated**	**1727422,0**
3.5	1	29.5	0	53.5	4	**value chi-square probability**	**1**
3.6	1	29.6	2	53.6	2		

Table 10. Main groups of fauna associated with the vermetid environments. x (x spp): Presence of a specific taxonomic group in a particular station (number of species per taxonomic group).

STATIONS/GROUPS	PORIFERA	CNIDARIA	POLYCHAETA	MOLLUSCA	CRUSTACEA	EQUINODERMA	VERTEBRATA (fish spp*)
ST1	x (2spp)			E. tulipus			x (9 spp)
ST3	x (1sp)	*Aiptasia* sp.		*E. tulipus*		x (1sp)	x (11 spp)
ST9	x (1sp)			*E. tulipus*		*Ophioderma* sp	x (15 spp)
ST12		*Leptogorgia alba*		x (1sp) *E. tulipus*	x (1sp)	*Diadema antillarum*	x (12 spp)
ST18	x (1sp)			*E. tulipus*			x (12 spp)
ST21	x (1sp)			*E. tulipus*			x (12 spp)
ST23	x (2spp)			*E. tulipus*		*Diadema antillarum*	x (8 spp)
ST29	x (2 spp)	x (1sp)		x (1sp) *E. tulipus*	*Mitrax* sp1 Anomura sp1 x (2 spp)	x (2 spp) *Ophioderma* sp1 Holothuria sp1 to sp5	x (8 spp)
ST31				*E. tulipus*		x (1sp)	x (8 spp)
ST45	x (1sp)			*E. tulipus*		x (1sp)	x (14 spp)
ST53	x (1sp)	x (1sp) *Aiptasia* sp.		*E. tulipus*		x (2 spp)	x (9 spp)
ST55		x (1sp)	x (1sp)	*E. tulipus*		x (1sp)	x (7 spp)

3.6. Discussion:

3.6.1. Taxonomy:

A new morphotype of the species associated with coral reef environments has been discovered in the Las Perlas Archipelago. Although Keen (1961), in the original description of the species as *Tripsycha (Eualetes) tulipu*s, mentioned the option of 'last whorl lax and uncoiled' in the final description it is mentioned only the complete coiled shells and only in rocky environments, due to the fact that the sampling was concentrated in Panama Bay. The inclusion of this new morphotype for coral reef environments extends the geographical and ecological distribution for this species. Observations in the field from the Pacific of Colombia collected by researchers at INVEMAR (Figure 17) show that there are similar shells in Gorgona Island. The above suggests that this new morphotype of the species could have a greater distribution, most probably throughout the whole TEP.

Figure 17. Shells (c.f. *E. tulipus*) from Gorgona Island, Pacific of Colombia taken by PhD student Mateo Lopez (INVEMAR-University of Gessen). Knife length: 25 cm.

3.6.2. Annual growth rate:

The observed large differences in annual growth rates are probably the result of the response of each organism to the environment. In stations where the vermetid was competing with corals that had a faster growth rate (e.g. *Pocillopora undata* in Pachequilla-station 1, *Pocillopora* spp in San Pedro-Station 29), most of the vermetids could not survive the competition and died (6 of 10 organisms in both stations were not found or had negative results due to dead and posteriori erosion, see Table 4). On the other hand, stations with the typical coral *P. damicornis* (e.g. Pedro Gonzales-station 55) permitted a better growth rate (8 organisms with positive results and an average growth rate for the station of 29.9cm). Bearing in mind that the branched corals of the genera *Pocillopora* and especially *P. damicornis* are the dominant species in Las Perlas Archipelago and the TEP reefs (Cortés 1997, Glynn and Mate 1997), the survival of *E.tulipus* in these type of reefs may represent basically the adaptation of the molluscs growth rate to the dominant coral growth rate in order to survive. Glynn and Macintyre (1997) mention that for *P. damicornis* the annual growth rate in Las Perlas Archipelago is 31mm/year and increases up to 39mm/year in other non upwelling Panamanian areas such as Uva Island. The authors also mention that the coral cover is higher on the fore-reef slope, where corals construct a wave-resistant and porous network of vertically oriented branches. Although this coral growth rates seem greater than that of *E. tulipus* rate, the corals (as frame) need to survive local mortalities, bleaching phenomena, and bioerosion, among other things. Thus, although individual coral colonies have a greater growth rate, the net growth rate for the whole reef per year is lower, with values of net coral accumulation in Saboga of 1.3m/1,000 years (Glynn and Macintyre 1997). Therefore, the molluscs need to compete for space during the reduced growth rate period (upwelling), when corals cannot compete favorably for space (Glynn 1993) and survive the periods of increased growth rates (non upwelling periods).

The vertical and uncoiled growth form in *E. tulipus* may be the ecological adaptation that the mollusc has found to compete for space with the main coral species and survive in this environment. When the molluscs find space or settle in coral reef environments dominated by other species (e.g. *Pocillopora undanta*) with greater growth rates, the mollusc simply cannot survive because of the higher growth rate of the coral, especially during the non upwelling months. Similar phenomena of competition for space have been reported in other sessile organisms associated with coral reef environments (Endean 1976, Sebens 1982, Glynn 1993, Márquez *et al*. 2006). In any case the growth

rate showed by the vermetids in las Perlas Archipelago is relatively high in comparison to the growth rate shown by other molluscs species associated with coral reef environments. An example is *Tridacna gigas*, which has growth rates of 16mm/year and 4mm/year for yourng and adults respectively (Aharon 1991).

In the case of the width growth rate, the annual value of 1.8mm/year (Table 4) represents the combination of three factors: increase in the thickness of the shell due to a new annual growth band and the epifauna that grows on the surface, plus the increase in the diameter of aperture of the tube as a response to the increase in the body mass. Bearing in mind that the theoretical size for the annual growth bands is around 121.4 µm (see Figure 11) one can say that the annual growth bands affect in a very small way the increase in the width of the shell, that was in cm. Therefore the width growth rate represents more the increase in the internal area of the snorkel (which is the response of the organism to the increase in its body mass) rather than a response of the organism to environmental variables.

3.6.3. Growth band patterns:

Although the estimation of the age of the shell was useful to identify the number of bands and to look for a pattern (e.g. 14 bands in figure 11), the bands are not homogeneous in size due to the fact that environmental variables (e.g. El Niño) could affect the thickness of some of the bands. For more discussion on growth band patterns and records of variables in the shells see chapter 9 (time series analysis).

3.6.4. Microhabitat :

The general results and the PCA analysis of this component show that healthy reefs (especially of the species *Pocillopora damicornis*), with a good coral cover, are essential for the settlement of *E. tulipus* and posteriori typical growth with the vertical tube. In the same way, the vertical tube will be useful only in reefs with slight slope (23° in average for Las Perlas Archipelago) where the coral branches grow vertically because it therefore allows the mollusc to reach the reef surface and access food directly in the water column. Thus, abundance and size of coral branches are factors that determine the physical structure of the reef in such a way that high abundances and cover are translated into a higher quantity of spaces that provide higher number of shelters (Barrios *et al.* 2002), which together with food seem to be the most important

factors for successful settlement for the mollusc. Sebens (1982) mentions that sessile organisms in coral reef environments will use these spaces according to the structure, scale, size and distribution of each species.

Due to the fact that each branched coral species has a different diameter of branches (Glynn and Mate 1997, Mate 2003b), the density of branches in a specific reef would be related to the coral species present in the area. Because the molluscs are growing with this typical tube only where there are coral reef environments, the average depth obtained for the microhabitats only represents the average depth of coral reefs in the area (depth affects only settlement and successful growth of the coral reef environment in which *E. tulipus* lives but it is not a limiting factor for the mollusc). This is the reason why, according to the PCA, depth was not a main variable to explain the microhabitat observed. The other morphotype (without the snorkel tube) has been reported in rocky areas of Panama Bay (Keen 1971) which are near the coast and therefore not deeper than 10m.

A very important factor is the protection that the environment offers. In the case of *E. tulipus* the protection is given by the density of coral branches. Very low density would mean over exposure to predators and very high density would mean very small opportunities for growth and access to resources. The average density value of coral branches obtained in the stations where the molluscs grow (12.6 branches/20cm) represents therefore the values around which *E. tulipus* is able to grow and survive. Values near this density were obtained in stations dominated by *Pocillopora damicornis*. This coral species has branches with thicknesses that vary according to the depth and grade of exposure (FAO, 1985) but, in the Las Perlas Archipelago, thicknesses are around 1-2cm, which means branches near the thickness of the average molluscs tubes and therefore this brings an extra protection because it helps the mollusc to mimetize within the coral branches. The results of the PCA show therefore that the most important factor in terms of microhabitat is the potential protection that the environments offer to the species, in this case represented by healthy areas of *Pocillopora damicornis* with a good density of branches, enough to make the mollusc's tube relatively invisible to potential predators.

3.6.5. Reef complexity:

The general results and PCA analysis for this component confirm the results of the microhabitat component in the way that only proper reefs, with a minimal reef thickness of 40cm and a good density of coral branches can offer good protection and potential for survival and reproduction to the mollusc. Coralline areas with very low thickness (poor vertical growth) are not considered proper reefs (Glynn and Macintyre 1977; Diaz *et al*. 2000) because they can not offer ecological space to other species. Bearing in mind that the results show that the abundance of vermetids on a specific reef would depend on these two components, density of coral branches and thickness, the third variable (abundance of vermetid) is really an expression of the combination of the first two variables. The above may be the reason why vermetids' abundance presented important but lower values in each of the first two components in the PCA.

3.6.6. Current reef structure:

The reefs where the vermetids (morphotype with snorkel) were found were dominated by branched corals of the genera *Pocillopora* and only in some stations (e.g st.12, Table 7) there were some coral heads in the bottom of the reef but there were no vermetids within those coral heads. The reason for this is that for the mollusc and other associated fauna of the coral reefs the most important factor for settlement is a physical reef structure that fulfills the requirements of the species in terms of protection and access for food (Barrios *et al.* 2002), in this case branched corals that provide first, space to access the water column for food, and second, a better protection against predators. Only when *E.tulipus* sp2 was within branched corals did it have the typical snorkel.

There was an evident difference in the requirement of coral cover between the general reef environment and the microhabitat. When the coral cover was estimated as one of the variables for the current structure of the whole reef the results were that the vermetids needed a minimal coral cover of 60% (see Table 7) but the requirements in coral cover increased when it came to microhabitat (minimum of 80%, see Table 5). The above means that although the vermetid can settle in reef environments that present different types of substrata (e.g. sand, dead coral, octocorals) it is essential for the species to be completely surrounded by coral branches in order to obtain good protection. A value of 60% in general life coral cover means healthy reefs, as can be seen in the description of the coral reef areas in Panama by Glynn and Macintyre (1977)

and more recently by Mate (2003b). The above explains the results of the PCA for this section in the way that a good and healthy reef, with high live coral covers (and therefore low dead coral cover) would offer a better space for the vermetids. Reefs dominated by *P. damicornis* (low number of coral species) were the preference for the vermetids.

3.6.7. Distribution pattern of vermetids:

Although Keen (1971) mentions that *E. tulipus* is a sessile molusc that prefers to live solitarily rather than in aggregations, the species was sometimes found in groups of even 9 organisms/quadrat. In those cases, as previously described by Keen (1971), the animals had the tips of the tubes in opposite directions. Freedman (1995) mentions that in coral reef environments where the succession has reached the maximum level or under intense disturbance, the new potential space available to begin a primary or secondary succession and restore the ecosystem is a result of death of other sessile organisms, especially the coral reef builders. Due to the high competitiveness for space in coral reef environments (Glynn 1993, Barrios and Lopez-Victoria 2001), the vermetid larvae need eventually to accommodate in what could be seen as aggregates to use in a maximum way the small spaces created. However, the statistical results confirm the assumptions of Keen (1971) in the way that their distribution seems to be more the result of a random availability of clear space for settlement and a tendency to solitary life. In this way, the general requirements of the species are the same no matter what the environment is, rocky or coral reef areas.

3.7. Conclusions

A new morphotype of *E. tulipus* is described associated with coral reef environments in Las Perlas Archipelago (Pacific of Panama). Observations in other areas show that its distribution could include the whole TEP.

The species showed an annual growth rate of 24mm with large variations among stations. These variations seem to be the response of the species to the surrounding environment, especially to the competition with the coral species that create the reef frame.

It was also possible to identify annual, monthly (spring tide bands) and daily growth bands. The monthly bands seem to be organized in two different groups: small and compact spring tide bands, related to the upwelling months, and bigger spring tide

bands produced by the mollusc during the rest of the year. The differences in the size of the annual growth bands may be a result of environmental factors that are affected in a larger scale, such as El Niño (ENSO).

In terms of microhabitat, the strong relationship between the presence of vermetids and healthy branched coral reefs expresses the requirements of the mollusc for environments that bring good protection against predators and a direct surface to the water column so they can access food. However the species is more flexible when it comes to the general reef environment. In this last case, the coral cover can be lower and the frame could include other benthic components apart from the branched corals. The only requirement is to be a proper reef frame, with a minimal vertical growth so as to permit good protection to the vermetid.

The distribution pattern of the species in the reef environments of Las Perlas Archipelago is, according to the statistical analysis, the result of a random availability of clear space for settlement and a tendency to solitary life. In this way, the general requirements of the species is the same no mater the environment, rocky or coral reef areas.

Chapter 4. Vermetids as archives of environmental change in the Pacific coast of Panama

4.1. Abstract:

Eualetes tulipus (Vermetidae) is a mollusc found in several areas within the Tropical Eastern Pacific-TEP, including Panama, associated with widespread reefs of branching corals (*Pocillopora*) and rocky areas. The mollusc produces a particularly long tube-shaped shell when it grows in reef environments. Therefore, growth patterns in the tube and variations in accumulated oxygen isotope and chemical markers (Sr, Mg, and Ba) were used to provide a potential record of environmental conditions (Sea Surface Temperature-SST and salinity) in a geographical area where it is only feasible to have occasional scattered instrumental recording stations and, in this way, to demonstrate the shells as potential "proxy" indicators of environmental change. The final goal with the comparisons against recent instrumental records was to provide a calibrated proxy that could be used to calibrate models used nowadays for quantification of environmental variability without the need for new instrumental records. Results from Panama shells showed a growth rate of 24mm/year and detectable annual, monthly and daily bands. X-ray diffraction and SEM analyses showed aragonite as the main shell component. Concentrations of oxygen isotopes in water showed similar results to those reported previously for the area. Comparisons between shells and water oxygen isotopes showed that accumulation of oxygen isotopes in shells are not in equilibrium with the sea water but are nearer to the isotopic equilibrium than corals. Analysis of trace elements (Sr, Mg, and Ba) and oxygen isotopes using different methodologies showed that there is detectable accumulation of the elements, in particular a positive relation between the Mg signal and SST and a negative relation between Sr signal and SST. There seems to be a negative relation between the Ba signal and salinity but it is necessary to perform more analyses in order to confirm this relationship. The relation between the trace elements and the records for SST and salinity are in general agreement with previous literature for other marine invertebrates. The results also showed a negative relationship between $\delta^{18}O/\ \delta^{16}O$ ratios and SST that is in general agreement with the previous literature for other marine invertebrates. Comparisons between methodologies for trace elements (ICP-OES, LA-ICP-MS, Ion Microprobe) showed ICP-OES as the best methodology to analyze annual growth bands in the vermetid, with a higher accuracy

and lower cost. When more detail and/or minimal amount of sample are needed Ion Microprobe is the most practical methodology. Besides, it gives the option to compare the results of the analysis with the band pattern from where they were collected. Comparisons between methodologies for oxygen isotopes showed that MS was good to analyze general growth band patterns, but Ion Microprobe was better to obtain more detail and to compare the results of the analysis with the band pattern from where they were collected.

4.2. Introduction:

Due to global warming and the increasing environmental deterioration in some areas, there is nowadays a substantial body of information on the analysis of historical marine environmental changes that has been provided by studying records throughout the world of skeletal deposition in tropical massive corals (e.g. Macintyre and Smith 1974, Aharon 1991, Guzmán *et al.* 1991, Tudhope *et al.* 1995, 1996, 2001, Guzmán and Jarvis 1996, Guzman and Tudhope 1998, Ferreira *et al.* 1998, Schrag 1999, Cardinal *et al.* 2001, Fallon *et al.* 1999, 2002, 2003, Watanabe *et al.* 2001, Swart and Grottoli 2003, Linsley *et al.* 2000, 2004). Moreover, in colder and tropical waters, other sessile organisms like the molluscs *Cerastoderma edule*, *Modiolus modiolus*, *Arctica islandica, Mercenaria mercenaria, Tapes philippinarum, Pecten maximus*, *Pinna nobilis* and *Tridacna* spp, have also shown good potential as proxy historical indicators (e.g. Jones *et al.* 1986, Richardson 1988, 2001, Romanck and Grossman 1989, Richardson *et al.* 2001, Aharon 1991, Bemis and Geary 1996, Watanabe and Oba 1999, Kennedy *et al.* 2001, Owen *et al.* 2002a, 2002b, 2002c, Elliot *et al.* 2003, Seed *et al.* 2000, Silenzi *et al.* 2004). Indeed, in the Tropical Easter Pacific-TEP region, where there is little reliable documentation of reef environments, and where there is a strong influence of ENSO on environmental conditions (SST, salinity-s‰, precipitation, nutrients, up-welling), molluscs like *Eualetes tulipus* provide numerous advantages over corals (especially the dominant branching corals *Pocillopora* spp in the region) that make it a new alternative and potentially good proxy indicator of environmental conditions, especially for water temperature and salinity (Table 11).

Table 11. Advantage of vermetid molluscs as proxy indicators of environmental variation with respect to branched corals in the TEP.

BRANCHED CORALS	MOLLUSCS
Structure: Identification of growth bands, isotope analysis and other temporal analysis are very difficult due to the fragmentary information that is collected from them since the branched nature of the coral structure confuses the chronology of skeletal deposition.	They secrete in general annual layers of carbonate which can be sub-sampled to obtain paleoenvironmental information with seasonal resolution (http://www.geos.ed.ac.uk/research/globalchange/ group4/Tridacna.html). They grow linearly without branches and therefore the temporal information collected is more continuous from the older base to the younger tip.
-There are **taxonomic and systematic difficulties** within the dominant group (*Pocillopora* spp) in TEP (Guzman Pers. Com.) that make it more difficult to understand the results (require further research to understand interspecies fractionation effects on analyses).	-Vermetids have been well studied as a mollusc family since the 60´s (Keen 1961, Keen 1971, Keen and Coan, 1974, Keen 1982). -There are advances on phylogeny/systematics through molecular analysis (Rawlings *et al.* 2001).
Corals precipitate skeleton out of **water isotopic equilibrium** (Pätzold 1992). The above cause problems in paleo-environmental reconstruction.	-Vermetids in the Panamanian area precipitate their skeletons (shell) almost in isotopic equilibrium with the surrounding sea water (per. Obs.) as do several other molluscs (Epstein *et al.* 1953, Elliot *et al.* 2003, Silenzi *et al.* 2004), and Tracers (e.g. Ba, Sr) may be controlled by T° in a more simple way than in corals (Cardinal *et al.* 2001, Fallon *et al.* 1999, 2002). If the above is proved, *E. tulipus* may yield a more robust method for historical $\delta^{18}O$ (T° /s‰) and $\delta^{13}C$ (metabolism in sea water) isotope analyses. It has also been shown that, in general, the geochemistry of mollusc shells can be controlled by environmental factors such as water temperature and water salinity http://www.geos.ed.ac.uk/research/globalchange/ group4/Tridacna.html).
Geographical distribution: *Pocillopora* spp are distributed in all TEP shallow water coral reef environments.	Vermetids are known in several rocky and coral reefs within TEP (Panama, Colombia, Ecuador, Costa Rica) and therefore probably occur throughout the whole TEP region. The molluscs in general unlike corals can be found in various ecological niches (tropics to high latitudes, coastal zones to deep ocean).

Current paleo-environmental research work in the United Kingdom that has used molluscs as proxy indicators includes the marine bivalves and paleo-environmental studies Group at Edinburgh University, coordinated by Dr. Mary Elliot (www.geos.ed.ac.uk/homes/melliot2), the work of Dr GM Henderson at Oxford University (NERC scholarship NER/S/A/2004/12388), and the group in biogeochemistry and palaeoceanography of the University of Wales (http://www.sos.bangor.ac.uk/research/php/theme.php?project=355).

Bearing in mind the work that has been done on this topic and the advantages of *Eualetes tulipus* (Mollusca: Vermetidae) with respect to the other natural archives in the area (the branched corals *Pocillopora* spp) the present work aimed to demonstrate the potential as a "proxy" indicator of temperature/salinity of this vermetid. The species has been identified in several areas within the Tropical Eastern Pacific (Ecuador, Colombia, Panama, Costa Rica) associated with widespread reefs of branching corals (*Pocillopora* spp) and within rocky areas. The mollusc produces a particularly long tube-shaped shell as it grows. Thus, the goal was to demonstrate that the growth patterns in the tube, and the variations in accumulated chemical markers (Sr, Mg, Ba, $\delta^{18}O$) in seasonal and annual growth cycles, calibrated against instrumental records collected from the same area, could be used to provide a record of environmental conditions in a geographical area where it is only feasible to have occasional scattered recording stations. The final goal with the comparison would be to provide a calibrated proxy record that could be used to calibrate models used for quantification of environmental variability prior to the instrumental records or without need of new instrumental records apart for those that already exist.

The hypothesis to be tested was: If mollusc growth is affected by natural environmental changes (e.g. temperature, up-welling), can shells of sessile molluscs (from isolated areas that lack good instrumental records on environmental data) be used as a quantitative proxy indicator of water mass conditions (e.g. ENSO) in the TEP during the last 10-30 years?

The aims associated with this hypothesis were:

1-To describe the growth rate and growth band patterns of the selected mollusc (*Eualetes tulipus*) in the Pacific of Panama (partially completed in chapter 3).

2-To determine the incidence and impact of SST and salinity during the last 10-30 years in the structural and chemical development (e.g. isotopes, tracers) of these vermetids.

3-To establish the correlation between the impact of change in water temperature (SST) and salinity on the vermetid population from Las Perlas (Panama) through comparison of results with instrumental records.

From the results an estimate would be made of the potential of *E. tulipus* as a proxy indicator of changes in water mass conditions for the area.

4.3. Study area:

The Pacific of Panama was chosen to address the potential of this mollusc as a proxy indicator of temperature due to the clear shifts in environmental conditions (especially the large changes in temperature and salinity) present in the area and associated with up-welling and ENSO conditions. These annual changes give a good basis for proxy measurement validation because they make the comparisons easier between the records and the potential proxy.

A general description of the TEP (including the area of Las Perlas Archipelago), their main coral reef formations and the general importance of TEP can be seen in chapter 2.

4.4. Methodology:

4.4.1. Field work:

Primary historical information spanning the last 10-30 years was obtained from shells collected primarily from Pedro Gonzales Island (station 55), and from another 3 coral reef environments in 2-7m water depth (stations 1, 12, 29). At each station (Figure 18, see Table 3) at least 1 shell was collected (tubes 15cm or longer that grow vertically as do the branches of coral) for growth band analysis, $\delta^{18}O$ isotopes, and trace element (Sr/Ca, Mg/Ca, Ba/Ca) analyses. The analyses were performed at Heriot Watt University. The goal was to correlate the primary information with seasonal and inter-annual changes in TEP water mass characteristics (water temperature and salinity) during the last 10-30 years (a period selected according to the reported lifetime of the species, up to at least 30 years). This period would be useful to identify recent SST anomalies, in this case the latest strong changes in temperature related to El Niño 1982-83/1986-87/1991-92/1997-1998 and the consecutive La Niña events, as well as changes related to seasonal up-welling periods. The aim of the growth band analysis, according to the methodology of Linsley *et al.* (2000), was to identify non-typical bands (those bands with growth during perturbations such as El Niño-La Niña), and typical bands (those ones that grow during “normal” years) that could be used to obtain more precise samples for oxygen and isotope analysis and for subsequent calibration against records of water temperature and salinity during the same period of time.

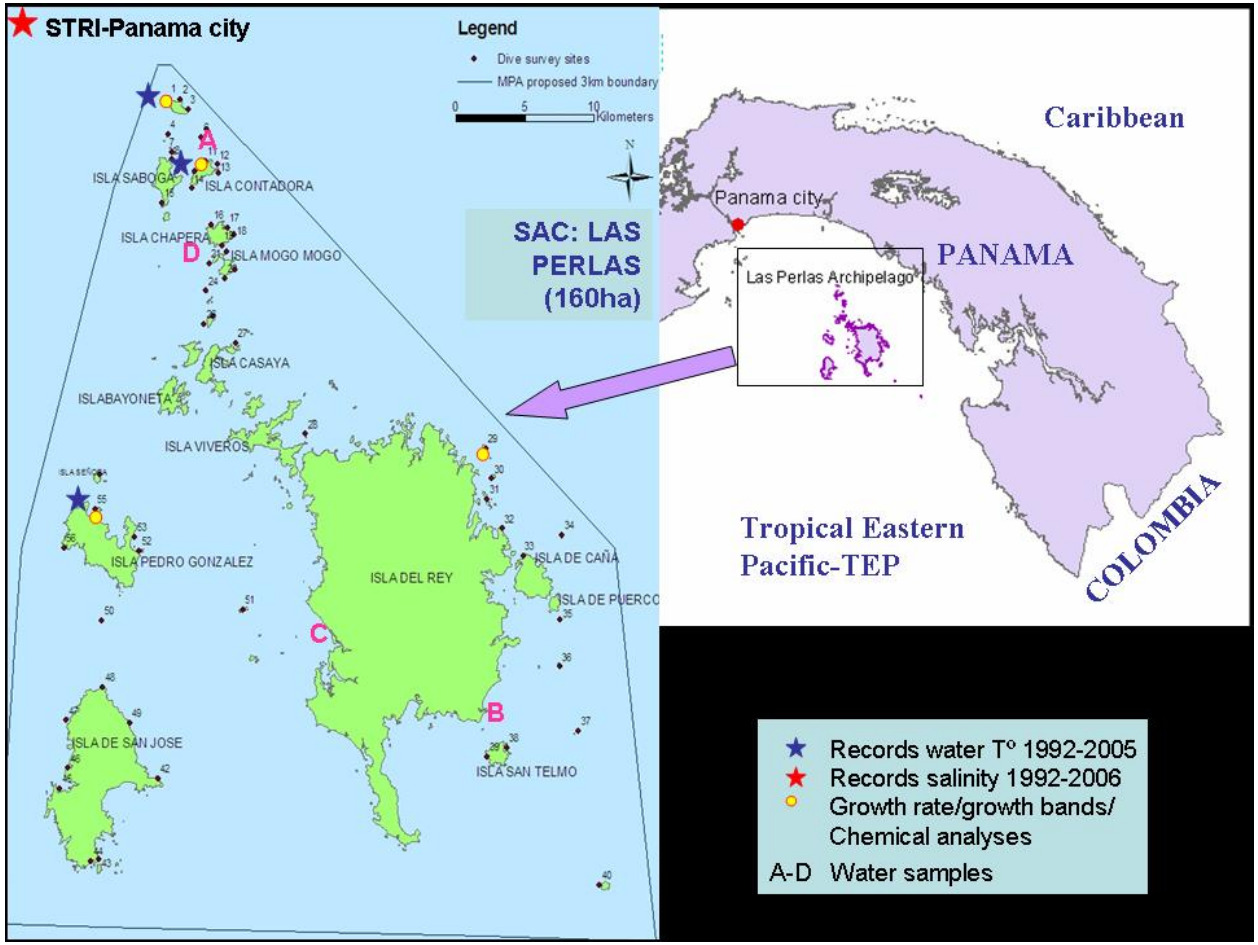

Figure 18. The Las Perlas Archipelago in the Pacific of Panama. Yellow spots are stations with collection of samples and primary ecological information (Stations 1, 12, 29, 55). Blue stars correspond to stations with secondary information (databases of SST for Pacheca, Saboga, Señorita). The red stars correspond to the station in STRI-NAOS (Panama city) with database for salinity. Letters A to D are the stations with collection of water samples for oxygen isotopes analysis. The other numbers (stations) are part of the general Darwin Project STRI-HW. Modified from GIS, Darwin Project STRI-HW University (2006) and **http://www.reef.org/data/database.htm**.

From the same four stations, and during the period August 2004-May 2005, information was also collected related to the longitudinal growth rate of the vermetids (objective 1) by monitoring the difference in size from 2004 when 10 shells in each station were measured and tagged *in situ*. The growth rate information was useful to confirm the analysis of growth bands in the shells. For more information about growth rates methodology see chapter 3 (methodology in field: page 45, statistical analysis: page 52).

In order to confirm the isotopic equilibrium of the vermetid samples with the seawater, 4 samples of surface water were collected from the Las Perlas Archipelago (see Figure 18: station 12-A, northeast of San Telmo-B, west of El Rey Island-C, station 21-D) to perform oxygen isotopes analysis. The samples were collected in black plastic containers (to reduce photosynthesis by phytoplankton or other reactions), kept frozen during the field trip and laboratory analysis in Panama and transported refrigerated to Scotland.

Historical secondary information relating to records of sea water temperatures from Las Perlas Archipelago and records of Salinity from Panama Bay, collected by scientific staff at STRI during the last 15 years (see Terrestrial-Environmental Sciences Program and CARICOMP of the Smithsonian Tropical Research Institute-STRI at http://striweb.si.edu/esp/physical_monitoring/download_orts.htm) were used for comparisons with the information collected during the field trips (objective 3).

4.4.2. Laboratory analysis:

a) Growth band patterns:

Due to the fact that the preliminary growth band analysis by using a modification of the methodology from Guzmán and Jarvis (1996) to identify the growth bands from the internal (by cutting the shell longitudinally and taking the x-ray from inside) and the external part of the shell did not show very accurate results in terms of a good pattern of bands (see Figure 7), it was essential to establish a new methodology to identify the growth bands in the mollusc. For details of the established methodology see laboratory analysis in Chapter 3 (page 50).

The final results from the monthly and daily growth bands were presented as a model of the type of growth in shells of this species. Following on from these results one typical shell with long snorkel was selected (tube PANAMA-2004-station55-shell8) for the next part of the analyses: trace elements and isotopes. In the case of the annual growth bands, the final picture was presented with the scale for the subsequent isotopes and trace element analysis.

b) Oxygen isotopes and trace elements:

In order to determine the incidence and impact of SST and salinity during the last 10-30 years in the structural and chemical development of *E. tulipus*, one typical shell (PAN-04-55-8) was selected to perform oxygen isotopes ($\delta^{18}O$/ $\delta^{16}O$ ratios) and trace element analyses (Sr/Ca, Mg/Ca, Ba/Ca ratios). Sub-samples from the internal part of the shell (non-typical and typical annual bands) to avoid epifauna or bio-erosion were taken according to the different techniques that were used during this work, drilling bands to obtain powder (trace elements analysis with Inductively Coupled Plasma Optical Emission Spectrometer ICP-OES, oxygen isotopes with stable isotope ratio Mass Spectrometer-MS, shell composition through X-Ray Diffraction), or preparing block samples from transverse sections of the shell for further analysis (trace elements and isotope analyses with the Ion Microprobe Facility IMF, trace element analysis with Laser Ablation Inductively Coupled Plasma Mass Spectrometer LA-ICP-MS, Scanning Electron Microscope analysis SEM, and Electron Backscatter Diffraction EBSC).

b.1) shell composition:

Electron Backscatter Diffraction –EBSC with the Scanning Electron Microscope-SEM and X-Ray Diffraction-XRD were performed at the Edinburgh Materials and Micro-Analysis Centre-EMMAC of Edinburgh University (http://www.geos.ed.ac.uk/facilities) in order to establish the composition of the inner layers.

The XRD (Philips PW1800, with phase analysis software SIROQUANT V2.5) is a powder diffraction analysis facility used for qualitative identification of minerals in all rock types. The facility has step and continuous scan capability and variable spinning. It uses different wavelengths to scan the sample and compares the sample with standard minerals to confirm its composition. For the analyses, 4 samples were collected (5mg shell powder), 2 from the inner layers (PAN-O4-55-5-2A, PAN-04-55-4-2A) and 2 samples from the external part of the shell (PAN-O4-55-5-2A*, PAN-04-55-4-2A*;

*:shows the samples from external part of the same shells from where internal samples were collected) to verify any potential change in general composition due to epifauna or bio-erosion. The samples were drilled at Edinburgh University micro-drill facility (Microscope LEICA GZ6, micro-drill, software MERCHANTECK E0).

The SEM (Philips XL30CP with PGT Spirit X-ray analysis) was a facility used to picture the annual bands inside the shell in order to use those pictures for the subsequent analyses (block samples: PAN-04-55-8, PAN-04-55-8-A/B, PAN-04-55-4-1B, PAN-04-55-5-1B, PAN-03-55-1 x 2, PAN-03-55-4 x2, PAN-04-55-7). The EBSC is a SEM technique that was used to confirm the results of the X-Ray diffraction analysis in terms of percent of calcite and aragonite. This technique is essential for further analysis like Ion Microprobe-IMF. The EBSC is a technique that analyses the diffraction patterns generated when an electron beam interacts with a crystalline sample. The diffraction patterns are imaged on a phosphor screen and analyzed by software (HKL Channel5 Electron Backscatter Diffraction-EBSD, windows environment) to determine the orientation of the crystal structure. The absolute crystallographic orientation, the preferred crystal orientations, and the size and grain boundaries were determinant factors to confirm the shell composition. Due to the resolution of the technique (~1 μm) and the size of most of the crystals observed during the analysis of the shells (< 1 μm), it was essential to do analysis from several individual points in which it was possible to identify any of the predetermined structures, calcite or aragonite. The only sample preparation required for SEM-ESBD was the production of a flat surface, through the inclusion of the samples in non-reactive resin and subsequent ultra polishing as explained in chapter 3 (3.4.2. laboratory analysis for growth band patterns, page 51). For the ESBD analysis only one shell was used (PAN-04-55-8, PAN-04-55-8-A/B).

b.2) Preliminary oxygen and carbon isotope analysis with MS:

Preliminary carbon and oxygen isotopes analyses, with sub-samples obtained from the external part of other shells (tubes PAN-04-55-1: 8 sub-samples, PAN-04-55-2: 14 sub-samples, PAN-04-55-3: 12 sub-samples), were performed to identify the best way for sub-sampling the shells (tubes) for the analysis and to confirm “no correlation” between carbon and oxygen isotopes concentrations. The samples were drilled at Edinburgh University micro-drill facility (Microscope LEICA GZ6, micro-drill, software MERCHANTECK E0) and analyzed according to the standard methodology (methodology for the VG PRISM III stable isotope ratio mass spectrometer-MS,

Tudhope 1999) at Wolfson Laboratory (School of Geosciences, Edinburgh University) for analysis of the external part of the shell as follows: Oxygen and carbon stable isotope analyses were performed on crushed 0.1-0.3 mg sub-samples, collected from the vermetid tubes. The carbonate powder was reacted with 100% orthophosphoric acid at 90 °C in an Isocarb automatic carbonate preparation system. The resulting CO_2 was then analyzed on a VG Isogas Prism III mass spectrometer.

In order to collect the samples for the analyses (to drill 0.1-0.3mg of powder per band) the vermetid tubes were set in resin (to give strength) in two main ways:

-mounted in resin blocks with the tube inside by using plastic moulds. Then a layer of the tube, with a longitudinal section of it, was cut to drill samples from the external part of the shell, from the youngest to the oldest monthly growth bands (Figure 19a).

-Tubes filled with resin and mounted in a plate to drill, from outside the tube, samples of the monthly growth bands (Figure 19b).

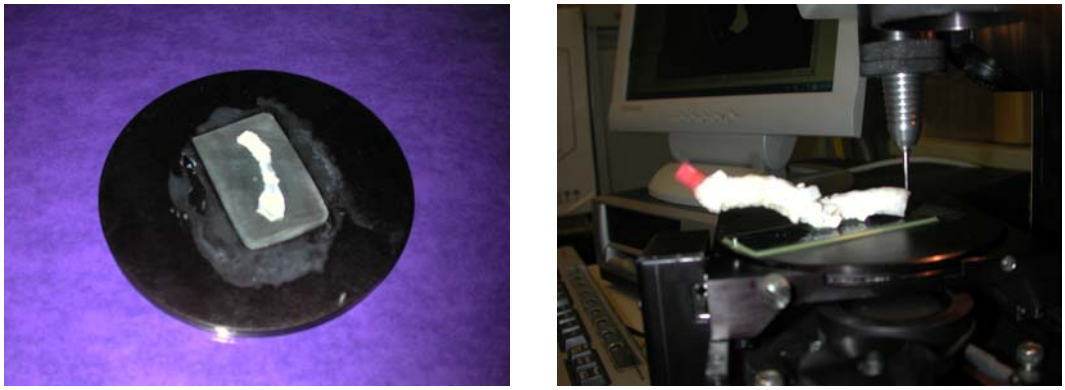

Figure 19. Example of the vermetid tubes from Panama (station 55) set in resin for the isotope analysis. a) Layer of resin block from sample PAN-04-55-1 (Panama-2004-station 55-tube 1). b) Tube of the same station (PAN-04-55-2) filled inside with resin.

To confirm the precision in the method and accuracy of the equipment 6 replicate samples of coral powder were analysed (COR1D: ±0.09‰ SD for $\delta^{13}C$ and ± 0.07 ‰ SD for $\delta^{18}O$; Mean value of $\delta^{18}O$ between December 2003 and March 2005 was -4.958 PDB, mean value of $\delta^{13}C$ between December 2003 and March 2005 was -0.688 PDB), and 12 analyses of marble powder (MAB2B: mean value of $\delta^{18}O$ between December 2003 and March 2005 was -2.475PDB, mean value of $\delta^{13}C$ between December 2003 and March 2005 was 2.424 PDB). The aim of using two standards was also to verify that the values between the standards remained constant. To prove the above (precision, accuracy, constancy), 5 samples of each standard were run within the samples in order to obtain the average and standard deviation for each standard. All carbonate isotopic values and standards were quoted relative to PDB (CO_2 derived from PDB $CaCO_3$ from

the rostrum of a Cretaceous belemnite, *Belemnitella Americana*, collected in the Pee Dee formation of South Carolina, international reference standard used to report variations in oxygen isotopes ratios; Francey and Allison (1998)).

b.3) Oxygen isotopes in sea water:

The analysis of the four surface water samples from Las Perlas Archipelago was performed at the East Kilbride NERC Mass Spectrometry Facility (Glasgow). The basic methodology is as follows (Epstein and Mayeda 1953, Socki *et al.* 1992, Fessenden *et al.* 2002):

Due to the high oxidation power of the water, the samples need to be equilibrated with CO_2 prior to the analysis. From each of the 4 samples (see Figure 17) 2ml of water were injected into a sealed vial (with a pierceable septum) which had been purged before with CO_2 (it is important to purge all the samples and standards within a batch with the same CO_2 bottle, as different CO_2 cylinders may have differing $\delta^{18}O$). Since the pressure in the vial is lower than atmospheric pressure, the water sample will be drawn directly into the vial. Then water samples, standards and blanks were left within each vial to equilibrate with the gas until the $\delta^{18}O$ and $\delta^{16}O$ have partitioned themselves at a constant level between CO_2 and H_2O. The vials were immersed in a constant temperature bath (25˚C). Due to the different equilibration times between the sea water samples (one week because they have more ion available), standards, and distilled water or blank (usually overnight), the vials were left for equilibration a full week to be sure that the CO_2 / H_2O mixtures were at equilibrium. The resulting CO_2 from the reaction was analyzed on the mass spectrometer (continuous flow isotope-ratio mass spectrometer). To confirm the precision in the method and accuracy of the equipment several samples of standards were also run (coral powder, Marble, mean values ± ‰ SD for $\delta^{18}O$).

The carrier for the analyses (to push the CO_2 through the system) was Helium (He). In this case He is flowing all the time and sample and standard gases simply enter the flow. The system controls the direction of He flow and thus the CO_2 is backflushed into the cleanup section, avoiding the use of cryogenics for transferral (liquid nitrogen in most of the cases). He continues to push the CO_2 through a Nafion trap (adsorbent material that traps any water that has been transferred along with the CO_2, thus the water can be pumped away separately later). Once pure, the CO_2 is transferred to the mass

spectrometer and analyzed as masses 44, 45 and 46. A CO_2 reference gas is also injected into the mass spectrometer (in light element stable isotope mass spectrometry) to measure the samples as isotope ratios relative to the reference gas, including the minimal contribution of $\delta^{17}O$, called "Craig" correction (Craig 1957).

The estimation of $\delta^{18}O$ in the seawater was made from the equilibrium fractionation factor alpha values [=($^{18}O/^{16}O$) CO_2 (g)/(^{18}O /^{16}O) H_2O (l), plus Craig correction] of the CO_2 in the seawater samples, due to the fact that it is possible to know the value of equilibrium for an element at a particular (constant) temperature. To access these values it was important then to keep the H_2O/CO_2 samples at a constant temperature for the entirety of the equilibration and the analysis. In order to obtain reproducibility for $\delta^{18}O$ of +/- 0.02‰ the temperature control system was kept within 0.1˚C of the required temperature by frequently measuring standard controls through the system. Prior to the analysis the water samples and blanks (solutions without sample and only standards, were used to calculate the LOD or accuracy of the method), were randomly distributed throughout the run to avoid possible sequence effects. The equipment was calibrated at the beginning, at the end, and during the analyses using the external set of standards in order to check the instrument performance. Each sample was analyzed three times against three reference gases (CO_2 reference gases) and the final results were expressed as mean values of each element in the sample. The isotopic values of the four samples were quoted relative to SMOW (Standard Mean Ocean Water, international reference standard used to report variations in water oxygen isotopes ratios) to be used in section b.6 to identify the theoretical values of the samples collected from the selected shell.

b.4) Trace element analysis with ICP-OES:

From the selected shell (PAN-04-55-8) 36 sub-samples were drilled at Edinburgh University micro-drill facility (Microscope LEICA GZ6, micro-drill, software MERCHANTECK E0), 6 samples every 24mm or estimated year (5 samples from 2004, 7 samples from 2003, and 6 samples for each year between 1999 and 2003), for the analysis of trace elements according to the standard methodology at the Scotish Associtation for Marine Science (SAMS). The samples were drilled from inside the shell to avoid epifauna. Bearing in mind that the mollusc creates new bands from inside, the youngest band would be in the internal surface and the tip of the shell (tube) would be thinner than the bottom. Therefore, in order to obtain bands from previous years it

was essential to collect first the sample of the last year or eliminate the first years before collecting a sample for a specific previous year. The thickness of annual bands had been calculated previously (see 8.4.3 growth band pattern results) as:

Thickness of annual bands= thickness of transverse section/age of the shell
Thickness of annual bands= 1614 μm/13.3=121.4
Thickness of annual bands= 121.4 μm

Thus, before collecting samples for the second year it was essential to drill and eliminate approximately 120 μm, 240 μm for third year, and so on. Due to the cylindrical surface of the shell (internal surface), the small thickness of the annual bands (121.4 μm), and the fact that the tip of the shells were thinner than their base, it was almost impossible to collect the amount of sample for the analysis (5mg) without mixing annual bands. Therefore, in order to obtain the minimal amount of sample it was decided to:

1) Collect the samples by drilling 4 consecutive lines of 60 μm (total depth: 240 μm) per sample.

2) Begin always from the surface and collect the samples from the tip to the bottom of the tube in the following way:

-For 5 samples from 2004: to collect the samples within the first 16mm of the tube (estimated growth rate for 8 months: January-August) only by drilling to collect each of the 5 samples.

-For 7 samples from 2003: 50 μm were eliminated before the collection. All the samples collected within the next 24mm with respect to previous year.

-For 6 samples from 2002: 210 μm were eliminated before the collection. All the samples collected within the next 24mm with respect to previous year.

-For 6 samples from 2001: 260 μm were eliminated before the collection. All the samples collected within the next 24mm with respect to previous year.

-For 6 samples from 2000: 300 μm were eliminated before the collection. All the samples collected within the next 24mm with respect to previous year.

-For 6 samples from 1999: 375 μm were eliminated before the collection. All the samples collected within the next 24mm with respect to previous year.

The above implies that the results were going to carry a general noise as a result of the overlapping of parts of the sample with the previous one. However, it was expected to see a general tendency or overall trend in the results.

The sample preparation, the digestions and dilutions were carried out in polypropylene (pp) sample tubes with LDPE stoppers. Prior to use, the tubes were soaked overnight in 2% aqueous nitric acid solution, then in deionesed (DI) water, and finally allowed to dry, inverted, in a test tube rack. The stoppers were not cleaned in this way due to difficulties in drying them again.

Each vermetid shell powder sample was weighed (5mg approx.) directly into a clean tube, and 0.5ml concentrated nitric acid (69% Merck Aristar, or equivalent grade) was added. The tube was stoppered, shaken, and left to digest at room temperature overnight. 4.5ml deionesed (DI) water was pipetted onto the digest, which was then stirred manually. The solution was allowed to stand for a further 2 hours before the final dilution was made. Then an aliquot of the digest was taken, which when diluted in nitric acid to 10ml, gave a total dilution factor of 10,000 (w/v). One aliquot of each of the 36 sub-samples was pipetted into a clean pp test tube (approximately 0.9 ml from each sub-sample and depending on the original weight of each of them), and 0.1 ml of 100µgml-1 Scandium solution was added (0.1ml of 100ppm Scandium-Sc + 0.25ppm of Lanthanum-La in 2%HNO3). This was made up to 10ml total volume with 2% aqueous nitric acid solution and mixed thoroughly. The solution was then transferred to the equipment to determine the Sr/Ca, Mg/Ca, Ba/Ca ratios.

The analyses were performed on an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) PerkinElmer Optima 4300DV Dual View. A dual view instrument allowed measurements to be made in both ways, axially (along the length of the plasma, here were analyzed Mg with Sc, and Ba with La) and radially (across the width of it, here were analyzed Sr with Sc, and Ca with Sc) in the same determination. This gives two quite different path lengths, which means that measurements of widely differing intensities (concentrations) can be made in the same determination. This facility is particularly useful due to the wide range of analytical concentrations, particularly between Ca and Mg. The calibration standards used for the analysis (Table 12) were prepared synthetically, in 2% aqueous nitric acid (2% HNO_3) from NIST-traceable single element solutions. A quadratic fit was used to generate the calibration

curves, and correlation coefficients were generally better than 0.999900. In order to minimize matrix effects caused by the high concentration of some elements (e.g. Ca) the method of standard addition was used.

Table 12. Calibration standards for the determination of Ca, Mg, Sr, Ba, and La in the vermetid samples. *Drift Control (slow deviation 2%).

Solution ID	Ca µg ml^{-1} (ppm)	Mg µg ml^{-1} (ppm)	Sr µg ml^{-1} (ppm)	Sc µg ml^{-1} (ppm)	Ba µg L (ppb)	La µg ml^{-1} (ppm)
Cal0	0.00	0.0000	0.0000	1.00	0.0000	0.25
Cal1	32.00	0.0400	0.2500	1.00	0.4000	0.25
Cal2	40.00	0.0800	0.5000	1.00	0.8000	0.25
Cal3	48.00	0.1200	0.7500	1.00	1.2000	0.25
Cal4	56.00	0.1600	1.0000	1.00	1.6000	0.25
Cal5	64.00	0.2000	1.2500	1.00	2.0000	0.25
DCS1*	40.00	0.1000	0.8000	1.00	1.0000	0.25

Due to the low concentration of Ba in the samples, a Ba standard dilution by approximately 1x 10^7 times (1 µg of sample per 1 L of 2% HNO_3) was prepared for measuring Ba. In the case of the other elements the standards were diluted by approximately 1x 10^5 times (1 µg of sample per 1 ml of 2% HNO_3). The solution "DCS1" did not form part of the calibration, but was used as a Drift Control Standard. This solution was designed to mimic the average sample composition as closely as possible, and was run at regular, specific intervals throughout every sample run. If the results lay out-with certain pre-determined values (Table 13), it was "failed" and repeated. If it failed a second time, the instrument was automatically recalibrated, and the DCS re-tested, before the sample run was resumed (the process was automatically activated if the established value, example 40 for Ca, changed in any way more or less than 2%). Initially, these pre-determined values were set at ±2%, but it was later found that the limits could be reduced to ±0.75% in some of the elements, without excessive failures (see Table 13). The instrument was therefore very stable over the time-scale of a sample run (6-7 hours), but any drift which did occur was controlled by the method outlined before.

Table 13. Failure limits for Drift Control Standard (DCS) from original determinations.

	Lower DCS limit, $ugml^{-1}$	Upper DCS limit, $ugml^{-1}$
Ca ±2%	39.20	40.80
Mg ±2%	0.0980	0.1020
Sr ±2%	0.7840	0.8160
Ca ±0.75%	39.70	40.30
Mg ±0.75%	0.0992	0.1008
Sr ±0.75%	0.7940	0.8060

Due to the fact that more than one isotope is available per element, several analytical and internal standard lines were monitored during the course of obtaining the data, as were some Argon lines to confirm continuous availability of carrier (Table 14). The final choices of emission lines were made on the basis of those which gave the least spectral interferences with the elements (lowest Radio Standard Deviation-RSDs and the "truest" values for the Drift Control Standard). The resulted calibration curve was applied through the ICP-OES analyses.

Table 14. Analytical and internal standard lines monitored during the ICP-OES analysis. *Elements selected as standard lines.

Element	Wave length (nm)	Type of element	Type of measurement
Mg	279.533*	Ion	Axial
Mg	280.271	Ion	
Mg	285.213	Atom	
Sr	407.771*	Ion	Radial
Sr	421.552	Ion	
Sr	460.733	Atom	
Ar	363.268		
Ar	420.069		
Ca	315.887	Ion	
Ca	317.993*	Ion	Radial
Ca	396.847	Atom	
Sc	357.634*	Ion: to analyze Mg	Internal standard for axial lines (Mg)
Sc	424.683*	Atom: to Analyze Sr&Ca	Internal standard for radial lines (Sr&Ca)
Sc	363.383*	Ion: to analyze Ca	Radial
Sc	357.253	Ion	
Sc	357.654	Ion	
La	257.933*	Ion: to analyze Ba	Internal standard for axial lines (Ba)

In order to monitor instrument sensitivity, an internal standard of ^{115}In and ^{209}Bi at a concentration of 10µg/L was added to all solutions, including the blanks. Prior to the analysis the vermetid samples, coral samples, and blanks (solutions without sample and only standards, used to calculate the limits of detection (LOD) or accuracy of the method), were randomly distributed throughout the run (EXCEL tool) to avoid possible sequence effects (to make experimental errors independent between samples). The coral samples were collected from Madang-Papua New Guinea in 1991 and have been run at SAMS as extra samples to calculate the precision of the entire process during the last 3 years in the same way as the coral standards for MS at Edinburgh University. The aim of using the coral standard was also to verify that the values between the standard remained constant. To prove the above, 5 samples of coral standard were run within the samples in order to obtain their average and deviation standard.

The equipment was calibrated at the beginning and the end of the analysis using the external set of standards (calibration curve). During the analyses of the elements (Mg, Sr, Ca, Ba) and in order to check the instrument performance, the ICP-OES was re-calibrated every 10 samples with a non-matrix matched multi-element solution at different concentrations (Mg and Sr at 25, 150, 500µg/L; Ca at 50, 400, 1000µg/L; Ba at 0, 0.4, 0.8, 1.2, 1.6, and 2.0 µg/L). Each sample was read three times. The final results were expressed as mean values of each element in the sample (%RSD). The value units for all the elements are COUNTS/second (arbitrary value that represent the scale of electricity produced by electrons from each element and that it is counted or estimated by the machine).

Previous works at SAMS has shown that the LODs (calculated from the series of acid blanks run throughout the analyses) can be calculated as 3 x standard deviation on the acid blanks for each separate session. LODs for a solid sample were produced from the instrumental LOD multiplied by the average dilution factor of the sample, as follows (Gordon 2002):

LOD= (3x standard dev.) x average dilution factor
Any analytical value below the LODs were set to zero.

The carrier for the analyses was argon at a flow rate of 1.06 L/min, with a drift or reduction of 1% during the running (the machine has a drift specification of 5-8%), showing that this was a successful procedure.

b.5) Trace element analysis with LA-ICP-MS:

Laser Ablation and Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) was carried out at SAMS (www.sams.ac.uk). The only sample preparation required for the analysis was the production of a flat surface, through the inclusion of the sample (a transvers section of selected shell PAN-04-55-8, replicate 2) in non-reactive resin and subsequent ultra polishing as explained in chapter 3 (3.4.2., laboratory analysis for growth band patterns, page 50). Prior to the laser-ablation, the sample was dipped in 2% HNO_3 for 15 seconds to remove surface contamination and then sonicated in deionized water for 5 minutes. The laser ablation analyses were carry out with a VG PlasmaQuad 3 ICP-MS coupled to a VG microprobe II pulsed Nd:YAG laser. The

procedure and operating conditions during the analysis were as follows (for further details see Gordon 2002):

1- A test was run (different laser lines) on the surface of certified standard glass to see the performance of the machine (Ln at 340nm, La at 210nm, U at 370nm, Pb at 210nm)

2- A test was run on a coral sample (MACS1) to confirm the performance of the phase (aragonite) in terms of Mg, Sr, Ca, Ba. This MACS1 is drained ground coral in a pellet form, with known concentrations of the main elements of interest. The pellet was prepared by an intra-laboratory survey by the United States Geological Survey-USGS and SAMS, and has been used by SAMS for almost 2 years.

3- To check reproducibility in the elements of interest (standardization) 3 holes (diameter hole 50 µm, depth 15 µm, output 60-70%, 20 seconds) in each material were run: glass (Ca, Cr, Fe, Co), Coral and vermetid sample (Ca, Mg, Sr, Ba). The results of this procedure, expressed as counts/second, showed that there was a constant area for Ca in the coral sample but it was very unstable in the vermetid sample, with a very small constant area, due to the fact that the sample was not absolutely flat for the analysis.

4- In order to obtain a smooth and flat surface a pre-ablation was performed (10 µm depth, laser thickness 200 µm, 1 line, output 45%, repeat 6 Hz) and step 3 was repeated several times until good performance was obtained for all the elements of interest. The last standardization of the sample within the pre-ablated surface was performed under the following conditions: 1 hole in each material (glass, coral, vermetid sample), diameter hole 75 µm (final diameter of the holes were 60 µm, less than the thickness estimated for the annual bands with the SEM: 121.4 µm), depth 20 µm, output 60%, repeat 10 Hz, 30 second per hole. The above conditions gave a good performance in the running of the elements, with %RDS that reduced from 25-35% to less than 9%, except Mg that kept values of RDS around 25%. Although the performance for Mg was not so good it was essential to keep those conditions to avoid compromising the results of the other elements.

5- The final run, that included the conditions obtained in step 4 (final diameter holes of 60 µm, running glass and coral standards at 15 µm depth and 70% output) was as follows:

-Pre-ablation sample

-Glass line

-2 Glass blanks (to order Argon for two holes without making the holes, not real sample)

-Coral line (MACS1)

-2 MACS1 blanks (same as in glass blanks)

-Line of 22 holes in vermetid sample on top of pre-ablated area (younger to older bands)

-MACS1 hole

-7 holes in vermetid sample

-1 MACS1 blank (to order Argon for 1 hole, not real sample)

-1 glass blank (to order Argon for 1 line, not real sample)

-2 glass holes (real holes)

-1 MACS1 blank (to order Argon for 1 line, not real sample)

-2 MACS1 holes (real holes)

Each sample was read several times by the machine. The final results were expressed as mean value of each element in the sample and its %RSD. The value units for all the elements were COUNTS/second (arbitrary values that represent the scale of electricity produced by electrons from each element and that it is counted or estimated by the machine).

The carrier for the analyses was argon at a flow rate of 1.06 L/min, with a general drift or reduction of 1.33% during operation (the machine has a drift specification of 5-8%), showing that this was a successful procedure. The drift was calculated from the data by comparing the counts per second (icps) obtained for the elements within the NIST 612 standard reference glass at the beginning (2 values for each element: 25Mg, 26Mg, 43Ca, 84Sr, 86Sr, 137Ba, 138Ba) and at the end of the analysis (2 values per element) and expressed as percent of drift or supression. The general drift was obtained from the average of the values obtained for each element.

b.6) oxygen and trace element analysis with the Ion Microprobe Facility-IMF:

The Ion Microprobe employs Secondary Ion Mass Spectrometry (SIMS) for the chemical analysis of small volumes of material (www.geos.ed.ac.uk/facilities). In SIMS the surface of the sample was bombarded under vacuum with a finely focused beam of primary ions (oxygen isotopes in the case of trace metal analysis and Cesium (Cs) for the oxygen isotopes analysis). The collision cascade results in the ejection and

ionisation of atoms and molecules from the surface layers of the sample. These secondary ions are accelerated into a double focusing mass spectrometer where they are separated according to their energy and mass/charge ratio before being detected.

There are three basic modes of operation:

- Point Analysis: A focused stationary beam is used to determine the composition at a point. The spot size is normally between 1 and 25 μm but is dependent on the application.
- Depth profiling: Scanning the primary ions over the surface of the sample causes the surface layers to be slowly eroded, revealing the compositional change with depth.
- Imaging (lines): The distribution of elements or isotopes may be imaged with a spatial resolution of about ~1um over areas up to 500 μm^2.

The Ion Microprobe can quantitatively analyze nearly all elements in the periodic table from H to U, but there are differences in sensitivity exceeding four orders of magnitude. However, for many elements the detection limits are in the PPB range. The instrument may also be used to determine the isotopic ratios of elements (e.g. C, O, Si, B, Li, S) to a precision of <0.05% in suitable material. The only sample preparation required for the analysis was the production of a flat surface as explained in chapter 3 (3.4.2., laboratory analysis for growth band patterns, page 51). The only difference with the previous methods is that for IMF a layer of gold paint was added to avoid electric current. During the analysis two preliminary test and two whole tests were run on the vermetid sample. The aim of the second whole test was to check reproducibility of the results.

<u>Oxygen isotopes ($\delta^{18}O/\delta^{16}O$):</u>

In order to determine and collect oxygen isotopes the mode of operation called Point Analysis was used with the following specifications: holes (created by the beam of Cs) of 40 μm in diameter, 5 μm depth, and distance between holes of 40 μm. In this case, the surface of the selected shell PAN-04-55-8 (with the golden layer) was bombarded under vacuum with a finely focused beam of Cs to obtain each sample. The values of oxygen isotopes from the samples were corrected back to an oxygen isotope standard with known $\delta^{16}O$ values (Sputtering with Cs enhanced the oxygen ion yield whereas sputtering with O enhanced the cation yield). The standard (oxygen) was measured 10 times at the beginning and end of each line of holes to obtain ratios ($\delta^{16}O/ \delta^{18}O$), averages, standard deviation and standard errors to monitor the precision of the procedure (40 measurements in total). Since oxygen was the major element there was no

blank as such but the faraday cups which do the current measurement were individually corrected for base line current and checked for gain differences between detectors.

The oxygen in each sample (holes of ~40 µm in diameter) was read for a period of 10 seconds by the detector (electron multiplier) and expressed directly as oxygen ratios ($\delta^{18}O/ \delta^{16}O$). The value units were counts/second (an arbitrary value that represent the scale of electricity produced by electrons from each element and that it is counted or estimated by the machine).

Trace elements (Mg/Ca, Sr/Ca, Ba/Ca):

In order to determine the selected trace elements the mode of operation called imaging was used. In this case, the carrier for the analyses was $\delta^{16}O$ ion beam, accelerated to 15 keV (combining 10.7 from the primary beam and 4.5 from the sample voltage gives 15.2KeV but it can be round to 15keV) with a beam current of 7nA as described in Allison *et al.* (2001). An energy offset of 75±20 eV was used and the spot size was ~5 µm in diameter and 5 µm depth, with distance between spots of 0 µm to create a line. Secondary ions were collected at masses of 44Ca, 88Sr, 26Mg, and 138Ba. To find suitable standards to calibrate the ion probe has proven difficult. Most carbonate standards are heterogeneous with respect to Sr making it impossible to ensure an accurate calibration. Despite this difficulty, analytical precision is good, and concentration differences between samples reflect true variations in geochemistry. Relative ion yields with respect to ^{44}Ca (Mg/Ca, Sr/Ca, Ba/Ca) were estimated, including a correction on carbonate standards (absolute concentration for OKA carbonatite as constants with the following values in counts/second: 688 for Mg/Ca ratios, 11967 for Sr/Ca, and 1296 for Ba/Ca) to provide independent estimates of absolute Sr/Ca from coral samples (Allison *et al.* 2001). The standard makes corrections for any ionisation differences between the elements. Typically, Mg ionization is about 0.3 of that in Ca, whereas Sr is very similar to Ca and Ba is about 0.7-0.8 time less than Ca. There were also run tests for each element as standard (^{44}Ca, $^{40}Ca2$, ^{26}Mg, ^{88}Sr, ^{138}Ba, $^{22.5}Na$=noise, ^{23}Na=background and it was used for calculations of ratios, ^{30}Si). Na was measured on the faraday cup (^{23}Na=background) as its intensity was too high for the electron multiplier. Corrections for the faraday cup background (^{23}Na) were made by using the count rate measured at mass $^{22.5}$ ($^{22.5}Na$=noise). The concentrations for this element were only an estimate as no standard was available. The Si was

measured to detect major contamination from sample preparation (i.e. holes or cracks in the sample and data were discarded if the Si content was too high).

The only blank estimated during the analysis was that for the background (detector background is typically less than 1 count/minute). Where count rates are above 10 counts/second (all the measures in this case) this blank can be ignored. No corrections were made for blanks on Mg, Sr or Ba.

Each sample (spots of ~5 µm in diameter) was read for a period of 10 seconds by the detector to obtain the amount of each element. The detector has a "faraday cup" that measures the current of atoms in general (less sensitive) and there is a background that if the value is "0" the machine will show a value of 40,000 as a way of checking the absence of atoms during that count. The final results were expressed as counts/second (arbitrary value that represent the scale of electricity produced by electrons from each element and that it is counted or estimated by the machine) of each element in the sample.

4.4.3. Statistical analysis:

a) Annual growth rates and growth band patterns:

See chapter 3 (page 52) for a description of the statistical analysis for this section.

b) Oxygen isotopes and trace elements:

b.1) shell composition:

Information collected during the XRD analysis was presented in graphics as percent of significant values of calcite and aragonite with respect to a minimal umbral (expressed by a dashed line).

b.2) Preliminary oxygen isotope analysis with MS:

The oxygen and carbon isotope values obtained from each shell (PAN-04-55-1, PAN-04-55-2, PAN-04-55-3) were organized in a database for subsequent Perasons correlation analysis between carbon and oxygen isotopes. The ideal result is to obtain "no correlation" between the isotopes. Positive or negative correlation between the isotopes implies a kinetic or biological effect of carbon isotope in the accumulation of

oxygen isotope (Adkins *et al.* 2003), which makes the oxygen signal more difficult to interpretae. The result of this analysis was essential to perform the subsequent oxygen and trace element analyses.

b.3) Oxygen isotopes in sea water:

The values of the four samples were used to obtain the mean $\delta^{18}O$ in water. This value was used to identify the theoretical values of $\delta^{18}O$ in the samples collected from the selected shell (see section b.6, page 105) and to confirm assumption of samples in isotopic equilibrium with the water environment.

b.4) Trace element analysis with ICP-OES:

The original results were expressed as weight percent of each element with respect to the total weight of the sample (e.g Ca= 39.66%, Mg=0.0171%, Sr=0.1594%) and its percent of radio deviation standard-%RDS that is the result of the variation between the three readings made for each element in each sample (e.g. Ca= 39.66% ±0.19%, Mg=0.0171%±0.134, Sr= 0.1594%±0.113%).

In order to eliminate the machine error, the mean value for each element, obtained from the 5 blanks, should be subtracted from the value obtained for each sample. However, this step was not necessary due to the fact that the values of the 5 blanks were approximately zero.

The values of each element (weight percent) were transformed to moles of each element/100g (e.g. moles of Ca/100g= weight obtained/weight of the element= 39.66 /40.078= 0.9896701) and then transformed to ratios of each element (Ba,Sr,Mg) with respect to Ca in ppm as follow:

$$\text{mmolMg/molCa (Mg/Ca in ppm)} = \frac{(\text{molesMg/100g}) * 1000}{(\text{moles Ca/100g})}$$

$$\text{mmolMg/molCa (Mg/Ca in ppm)} = \frac{(\text{weight Mg obtained/weight Mg}) * 1000}{(\text{weight Ca obtained/weight Ca})}$$

$$\text{mmolMg/molCa (Mg/Ca in ppm)} = \frac{(0.0171/24.305) * 1000}{(39.66/40.078)}$$

$$\text{mmolMg/molCa (Mg/Ca in ppm)} = (0.0007040*1000/0.9896701)$$

$$\text{mmolMg/molCa (Mg/Ca in ppm)} = 0.7113$$

The new ratio standard deviation of this proportion was obtained as follows:

%RDS Mg/Ca= $\sqrt{((\%RDS\ Ca)^2 + (\%RDS\ Mg)^2)}$

%RDS Mg/Ca= $\sqrt{(0.19^2 + 0.0171^2)}$

%RDS Mg/Ca= 0.23

The new ratios in mmol/mol (Mg/Ca and Sr/Ca in ppm), µmol/mol (Ba/Ca) and their %RDS were presented graphically as ratios vs time. The relationship between Mg/Ca and Sr/Ca with temperature during the period 1999-2004 was quantified by linear regression of the ratio measurements against the Sea Surface Temperature-SST records collected for Las Perlas by scientific staff at STRI. This calibration involves the next assumptions (Villierts *et al.* 1994, Fallon *et al.* 2003):

- Minimal Sr/Ca ratios correspond to maxima SST (negative relation)
- Minimal Mg/Ca ratios correspond to minimal SST (positive relation)
-Temperature and salinity are the main controls but temperature is the primary control

The relationship between Ba/Ca and salinity during the last 15 years was also quantified by linear regression of the Ba/Ca against the salinity records collected for Panama Bay by scientific staff at STRI. This calibration involved the following assumptions (see Tudhope *et al.* 1997):

-Minimal Ba/Ca ratios correspond to high salinity records (negative relation)
-Salinity (including rainfall) and riverine discharges are the main controls but salinity is the primary control.

The values and "shape" of each graph or element (that were ploted against SST or Salinity, respetively) were then compared with those obtained for the same elements with the other techniques (see b.5 and b.6) to identify the best methodology to be used with this mollusc species.

<u>b.5) Trace element analysis with LA-ICP-MS:</u>

The original results were expressed as counts of each element (e.g 43Ca= 1214579.2, 26Mg=31202.08, 86Sr=1932559.5, 138Ba=43786.58) and its %RDS that is the result of the variation between the readings made for each element in each sample (e.g. 43Ca=1214579.2±6.291, 26Mg=31202.08±9.921, 86Sr=1932559.5±7.215, 138Ba=43786.58±11.226).

In order to eliminate machine error the mean value of the element obtained from the 5 blanks was subtracted from the value obtained for each sample (e.g new 26Mg

value=31202.08-570.0587= 30632.02) and then transformed to ratios of each element (Ba,Sr,Mg) with respect to Ca in ppm as follows:

Mmol 26Mg/mol 43Ca (Mg/Ca in ppm) = new 26Mg *1000/ new 43Ca

Mmol 26Mg/mol 43Ca (Mg/Ca in ppm) = 30632.02 *1000/1213277.42

Mmol 26Mg/mol 43Ca (Mg/Ca in ppm) = 25.24733

The new ratio standard deviation of this proportion was obtained as follows:

%RDS 26Mg/43Ca= $\sqrt{((\%RDS\ Ca)^2 + (\%RDS\ Mg)^2)}$

%RDS 26Mg/43Ca= $\sqrt{(9.9201^2 + 6.291^2)}$

%RDS 26Mg/43Ca= 11.74

The new ratios in ppm (mmol/mol of Mg/Ca, Sr/Ca, Ba/Ca) and their %RDS were analyzed graphically as ratios vs time. The relationship between Mg/Ca and Sr/Ca with temperature during the last 15 years was quantified by linear regression of the ratio measurements against the Sea Surface Temperature-SST records collected for Las Perlas by scientific staff at STRI. The relationship between Ba/Ca and salinity during the last 15 years was also quantified by linear regression of the Ba/Ca against the salinity records collected for Panama Bay by scientific staff at STRI. The calibration between Mg and SR with SST, and Ba with salinity involved the assumptions previously presented for ICP-OES analysis (see page 103).

The values and "shape" of each graph or element (that were ploted against SST or Salinity, respetively) were then compared with those obtained for the same elements with the other techniques (see b.4 and b.6) to identify the best methodology to be used with this mollusc species.

b.6) oxygen and trace element analysis with Ion Microprobe Facility-IMF:

Oxygen isotopes ($\delta^{18}O/\delta^{16}O$):

The original results, in this case units of the oxygen ratios ($\delta^{18}O/\ \delta^{16}O$, in counts/second), were transformed to SMOW with a formula that includes a correction to eliminate the machine error by using the average value of the 40 measurements of the standard ratio ($\delta^{18}O/\delta^{16}O$) in counts /second and in SMOW as follows:

FORMULA No.1:

Δ $^{18}O/$ ^{16}O in SMOW= ((sample $^{18}O/$ ^{16}O in counts/sec. / average $^{18}O/$ ^{16}O of 40 measurements of standard in counts/sec.) * (Value or UWC carbonate standard +1000)) -1000

Therefore, if we have a value of $^{18}O/$ ^{16}O (counts/sec)= 0.002050116, the same value in SMOW was:

Δ $^{18}O/$ ^{16}O in SMOW= ((0.002050116/0.00204108) * (23.28 +1000))-1000

Δ $^{18}O/$ ^{16}O in SMOW= 27.81

Then the values in SMOW were transformed to PDB with the following formula:

FORMULA No. 2:

Δ $^{18}O/$ ^{16}O in PDB = 0.97002* Δ $^{18}O/$ ^{16}O in SMOW -29.98

The values given in the equations are known values for the carbonate standard used (UWC carbonate), for which the difference between it (in PDB) and SMOW has been determined by bulk analysis as 29.98.

Thus, the previous value in SMOW (27.81) would correspond in PDB to:

Δ $^{18}O/$ ^{16}O in PDB = 0.97002* 27.81 -29.98

Δ $^{18}O/$ ^{16}O in PDB = -3.00

The new oxygen ratios in PDB were analyzed in a descriptive way (graphic) as ratios vs time. The relationship between oxygen isotopes values and SST during the last 15 years was quantified by linear regression of the ratio measurements against the SST records collected for Las Perlas by scientific staff at STRI.

In order to confirm assumptions of isotopic equilibrium between the oxygen isotopes in the shells and the values for oxygen isotopes obtained from water samples collected in the area, a modification of the Epstein *et al.* (1953) formula presented by Grossman and Ku (1986) was used. From this formula the theoretical value of $\delta^{18}O$ in aragonite (for the shell) was obtained as follows:

Y= a - bX

SST (°C)= 21.8 - 4.69 ($\delta^{18}O$aragonite- $\delta^{18}O$water)

(See formula 3 for molluscs in Grossman and Ku 1986)

$SST - 21.8 = -4.69\ (\delta^{18}O_{aragonite} - \delta^{18}O_{water\ in\ VSMOW})$

$$\frac{(SST - 21.8)}{4.69} = -\delta^{18}O_{aragonite} + \delta^{18}O_{water\ in\ VSMOW}$$

$$\frac{(SST - 21.8)}{4.69} - \delta^{18}O_{water\ in\ VSMOW} = -\delta^{18}O_{aragonite}\ (-1)$$

$$\frac{(SST - 21.8)}{-4.69} + \delta^{18}O_{water\ in\ VSMOW} = \delta^{18}O_{aragonite}$$

$$\delta^{18}O_{aragonite} = \frac{SST-21.8}{-4.69} + \delta^{18}O_{water\ in\ VSMOW}$$

And including the additional correction to changes values in water from VSMOW to VPDB (see page 62 in Grossman and Ku 1986: $\delta^{18}O_{w\ in\ VPDB} = \delta^{18}O_{w\ in\ VSMOW} - 0.27$):

FORMULA No. 3:

$$\delta^{18}O_{aragonite} = \frac{SST-21.8}{-4.69} + \delta^{18}O_{water} - 0.27$$

Water samples were collected to obtain mean $\delta^{18}O$ in water (value= -0.32 see section b.3 in methodology and results).

*Small variations in the Grossman and Ku (1986) formula are presented in Böhm *et al.* (2000) but the relation between the theoretical values from the shell (in PDB) and the water (in VSMOW) does not include the correction (-0.27).

The new formula (formula No.3) was used to obtain the mean, maximum and minimum <u>theoretical</u> values in order to know the potential range of variation in the values obtained with the analyses (theoretical $\Delta\delta^{18}O$ aragonite= $\delta^{18}O_{aragonite}$ max.- $\delta^{18}O_{aragonite}$ min.) by replacing SST with the:

a) Average monthly SST for Las Perlas Archipelago between June 1992 and February 2005 collected by STRI.

b) Maximum monthly SST for Las Perlas Archipelago between June 1992 and February 2005 collected by STRI.

c) Minimum monthly SST for Las Perlas Archipelago between June 1992 and February 2005 collected by STRI.

To identify historical statistical differences in the values (potential El Niño, La Niña years), other 3 values per year were obtained from the slope of the linear equation formula (slope=1/b). The new values, plus the original obtained from the analysis were used for the comparisons with SST.

Trace elements (Mg/Ca, Sr/Ca, Ba/Ca):

The original results were expressed as counts of each element (e.g 23Na background=1151841.7, 26Mg=1267.52, 30Si=1341.03, 48Ca=56776.2, 88Sr=77770.8, 138Ba=167) and then transformed to ratios of each element (Ba,Sr,Mg) with respect to Ca in ppm (e.g 23Na/Ca (ppm)=Mmol 23Na background* constant for the element/mol 48Ca). 30Si/Ca ratios were used to confirm precision in the machine (large values mean errors in calculations).

The new ratios in ppm (Mg/Ca, Sr/Ca, Ba/Ca) were analyzed graphically as ratios vs time. The relationship between Mg/Ca and Sr/Ca with temperature during the last 15 years was quantified by linear regression of the ratio measurements against the SST records collected for Las Perlas by scientific staff at STRI. The relationship between Ba/Ca and salinity during the last 15 years was also quantified by linear regression of the Ba/Ca against the salinity records collected for Panama Bay by scientific staff at STRI. The calibration between Mg and SR with SST, and Ba with salinity involved the assumptions previously presented for ICP-OES analysis (see page 103).

The values and "shape" of each graph were compared with those obtained for the same elements with the other techniques (see b.4 and b.5) to identify the best methodology to be used with this mollusc species.

c) Comparison of results from this work with instrumental records:

Appropriate statistical techniques (e.g. stationary time series analysis with the Autoregressive Moving Average model-ARIMA – Linsley *et al.* 2000, 2004, or a more simple regression analysis) were used to analyze the results from the previous section (b.4, b.5, b.6) and related to the historical environmental information records (SST, salinity) that have been gathered during the last 10-15 years within the area.

In the case of the SST, data sets from 3 different stations in the Las Perlas Archipelago (Pacheca Island1995-2005-6m depth, Senorita Island 2001-2004-12m depth, Saboga

Island 1992-2002-1m depth) were provided by the Terrestrial-Environmental Sciences Program and CARICOMP of the Smithsonian Tropical Research Institute (http://striweb.si.edu/esp/physical_monitoring/download_orts.htm). Information related to salinity was obtained from Panama Bay (wet laboratory at STRI-NAOS) due to the absence of databases for Las Perlas Archipelago. In order to analyze the different ENSO episodes including during the study period (EL NIÑO 82-83, 86-87, 91-92, 97-98 and subsequent La Niña events) and look for correlations between ENSO events and the different parameters collected during the current study, the results of water oxygen isotopes analysis were compared with those reported by other authors (value oxygen isotope, year, area) for the area at the Goddard Institute for Space Studies-NASA (http://data.giss.nasa.gov/o18data/, Schmidt *et al.* 1999). Results of SST database from STRI were compared with those reported by other authors for the area at NOAA (http://www.emc.ncep.noaa.gov/research/cmb/sst_analysis/) and NASA (http://poet.jpl.nasa.gov/). The information of SST in the Panamanian Pacific collected at NOAA was organized as monthly values (1990-2005) for the analysis.

4.5. Results:

The mollusc was first described as *Eualetes tulipus* by Keen (1971, 1982). For more details about identification by traditional taxonomy, confirmation by molecular analysis and general ecology see chapter 3.

4.5.1. Annual Growth rate:

Bearing in mind that calculations for growth rate were based only on those shells found alive in 2005 (positive results, including zero growth), the average annual longitudinal growth rate of *E. tulipus* in Las Perlas Archipelago for the period 2004-2005 was estimated as 24.2(±20.9) mm/year. Table 4 (see chapter 3) shows the average longitudinal and width growth rate (± standard deviation) for each of the 4 selected stations during the nine month period and estimations of annual growth rate in Las Perlas Archipelago for the period 2004-2005. From the table it is evident that there was a big difference in growth between samples and stations, including null or negative results that correspond to molluscs found dead and with deterioration (bioerosion) during the second year, especially in station 1 (Pachequilla) and 29 (San Pedro). Due to the larger variations in the measurements for width growth rate (both variables should

have a similar behaviour) only longitudinal growth rate was used for subsequent analysis of oxygen isotopes and trace elements.

Although estimations of annual longitudinal growth rate for the whole archipelago showed values around 24mm/year, the growth of the species was very different according to the station in which the molluscs were living (see Figure 10 in chapter 3). Station 12 presented the highest longitudinal growth rates and standard deviation. On the other hand, station 55 was more stable (lower standard deviation) but showed the lowest growth rates. The differences between stations could be explained in terms of the requirements of the species with respect to the general environment, rocky areas or coral reefs (see results for reef complexity and current reef structure in chapter 8). The large differences in longitudinal growth rate within the stations (see Table 4, standard deviations per station) also show an effect of the microhabitat on the growth rate of each organism. See chapter 8 for results of PCA analyses developed to identify the most important variables for *E. tulipus* with respect to microhabitat, reef complexity and current reef structure.

4.5.2 Growth band patterns:

Laboratory analysis (stereoscope, microscope and Scanning electron microscope-SEM) with sections of shells collected in 2004, free of epifauna, showed detectable daily, monthly (a couple of spring tide bands per month) and annual bands (see Figures 11 and 12 in Chapter 3, page 61).

From the identification of the monthly bands it was also evident that there were groups of compacted monthly bands (months December to April, the up-welling period in the Pacific of Panama) and groups of spaced bands that corresponded to the other months (see Figure 11 in Chapter 3).

Daily bands were identified only through the microscope (5X with normal microscope or under good stereoscope). In this case, the couple of visible brown bands per month corresponded to two groups of three to four minor brown bands that were created during the spring tides (two per month), and between each group of minor brown bands there were twenty to twenty-four white bands (see Figure 11A in Chapter 3). The total number of daily bands between a group of dark daily bands and the next one was around

28, which corresponds more to a lunar period than a calendar month. The surface of the shells, from the bottom to the tip of the snorkel, presented valleys and crests. Thus every valley was created by a group of white daily bands (approximately one valley per month) and the crests by the two groups of daily spring tide bands that are formed approximately every month.

The images of the annual band pattern obtained with the SEM (Figure 20A) also show that there are two types of annual bands per year:
-small and white bands that may be created by the group of compacted monthly bands related to the up-welling period in the area, and
-Larger and darker bands that may be created by the group of spaced monthly bands formed during the rest of the year.
However, this pattern was more evident after applying a gold-paint layer on the selected shell (selected transverse section: PAN-04-55-8) for the subsequent analysis with the Ion Microprobe (Figure 20B).

Based on the estimated age of the selected organism at the moment of collection (see growth band patterns in chapter 8, page 64), approximately 14 years, and the thickness of the transverse section (1614 µm), it was possible to estimate the theoretical size of annual bands in the transverse section (1614 µm/13.3=121.4 µm each year). The theoretical size of the annual bands and the number of years worked only as a guide to compare with the number of bands observed in the transverse sections. Thus, it was possible to identify 14 annual bands (1991-2004) in the selected shell (PAN-04-55-8), including a small section in the youngest part of the shell (left) that would correspond to part of 2004.

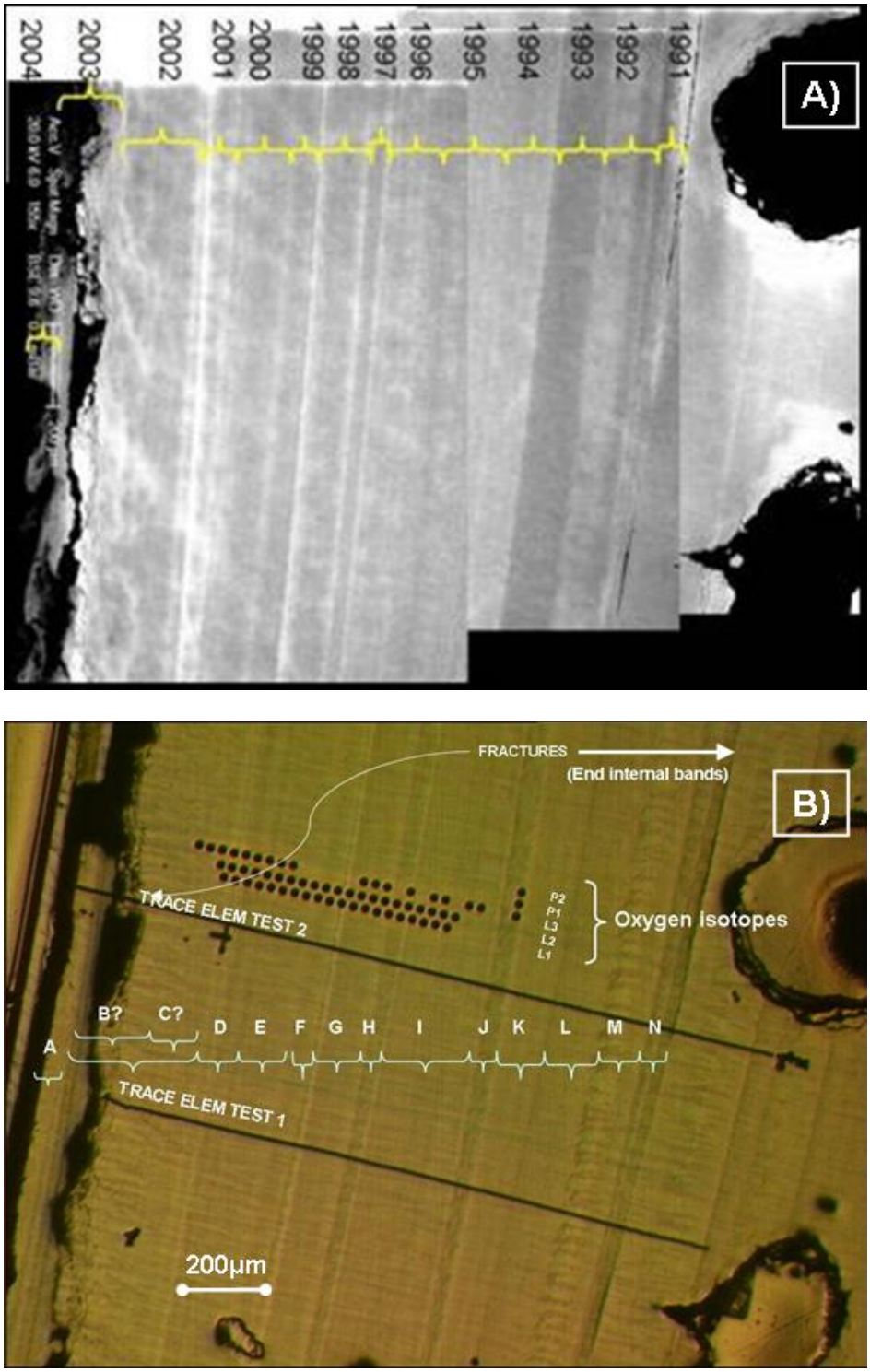

Figure 20 Band patterns for sample PAN-04-55-8 (transverse section) in which there are evident 14 annual bands, including a small section on the left that corresponds to 2004. A) Band pattern under SEM. Yellow brackets indicate the width of the 14 annual bands. Left side of the image corresponds to the internal part of the snorkel (see Figure 6 for details of cross sections), where the youngest band (2004) was growing. Right side of the image corresponds to the end of internal layers in the shell (holes are bioerosion by epifauna), where the oldest band was deposited. B) Band pattern after Ion Microprobe Analysis-IMF in which there are evident 14 annual bands (letters A to N, 14 years) and the scars of the trace element and isotopes analysis on the surface. Trace element test 1 and 2 correspond to the lines made by the beam during the test for trace elements. L1, L2, L3, P1, and P2 correspond to the lines of dots and extra dots made during the oxygen isotope analysis.

4.5.3 Shell composition:

Results of X-ray diffraction-XRD analysis on samples obtained from internal (PAN-04-55-5-2a-2, PAN-04-55-4-2a-3; Figure 21b) and external parts of the shells (PAN-04-55-5-2a*-4, PAN-04-55-4-2a*-1; Figure 21a) showed aragonite as the main component or phase. In the analysis, only peaks higher than the umbral (dashed black lines) were considered significant. Only one significant peak in each of the external samples (orange arrows in Figure 21a) and one peak in one of the internal samples (orange arrow in Figure 21b) included calcite as the main component (but with low values). Thus, the analyses showed a composition of 99.9% aragonite and 0.1% calcite. This calcite could be part of bioeroders (internal part of shells) or epifauna attached to the external part of the shells. Therefore, EBSC with the SEM analysis was performed to confirm 100% of aragonite. Results of this last analysis showed that in all the possible cases in which a crystal structure was evident (because crystals in the *E. tulip*us shells showed to be in general smaller than the minimal equipment resolution, <1μm) the composition of the crystals was aragonite.

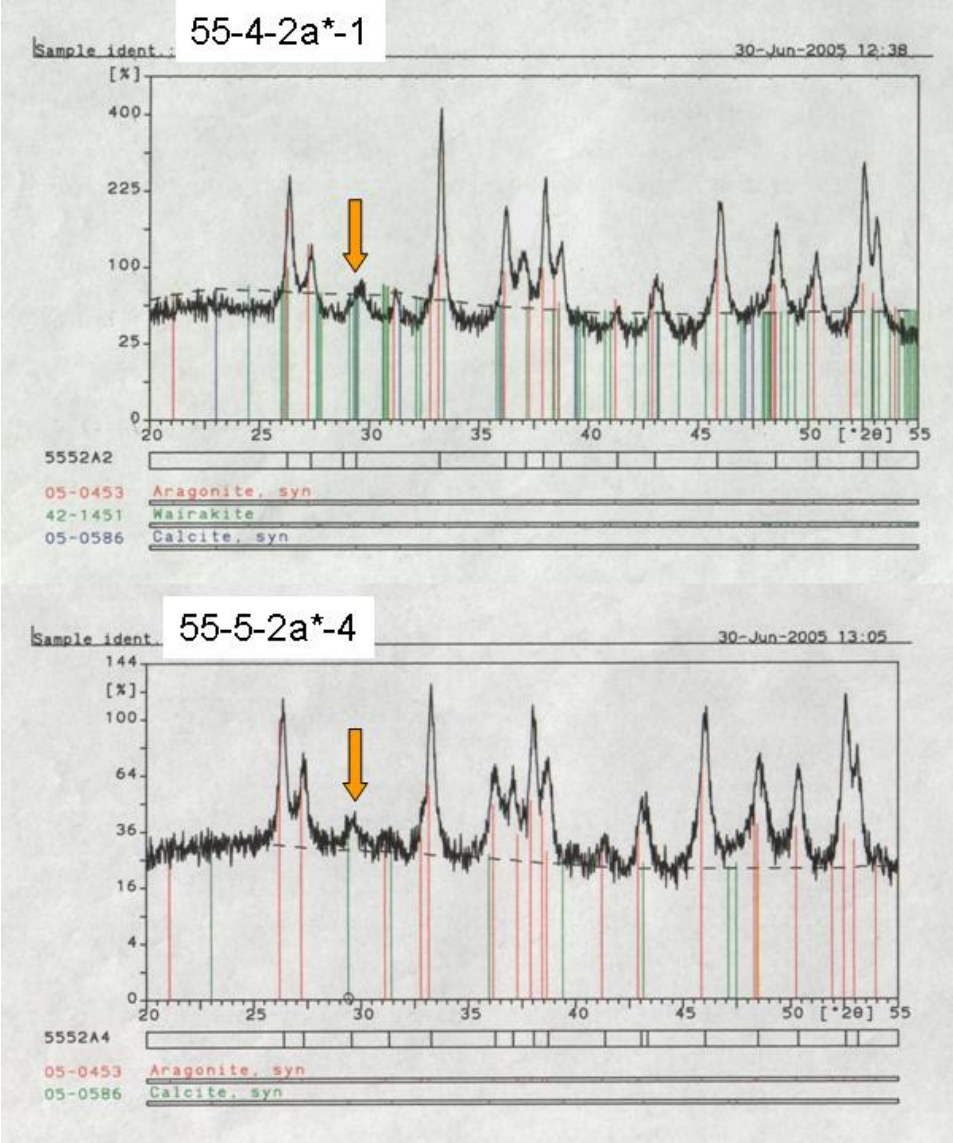

Figure 21a. Results of X-ray diffraction-XRD analysis for external parts of the shells (samples PAN-2004-55-5-2a*-4 and PAN-2004-55-4-2a*-1). Red lines represent peaks of aragonite. Green lines represent peaks of calcite. Dashed lines represent minimal umbral to consider significant values. Orange arrows show significant peaks of calcite in each shell.

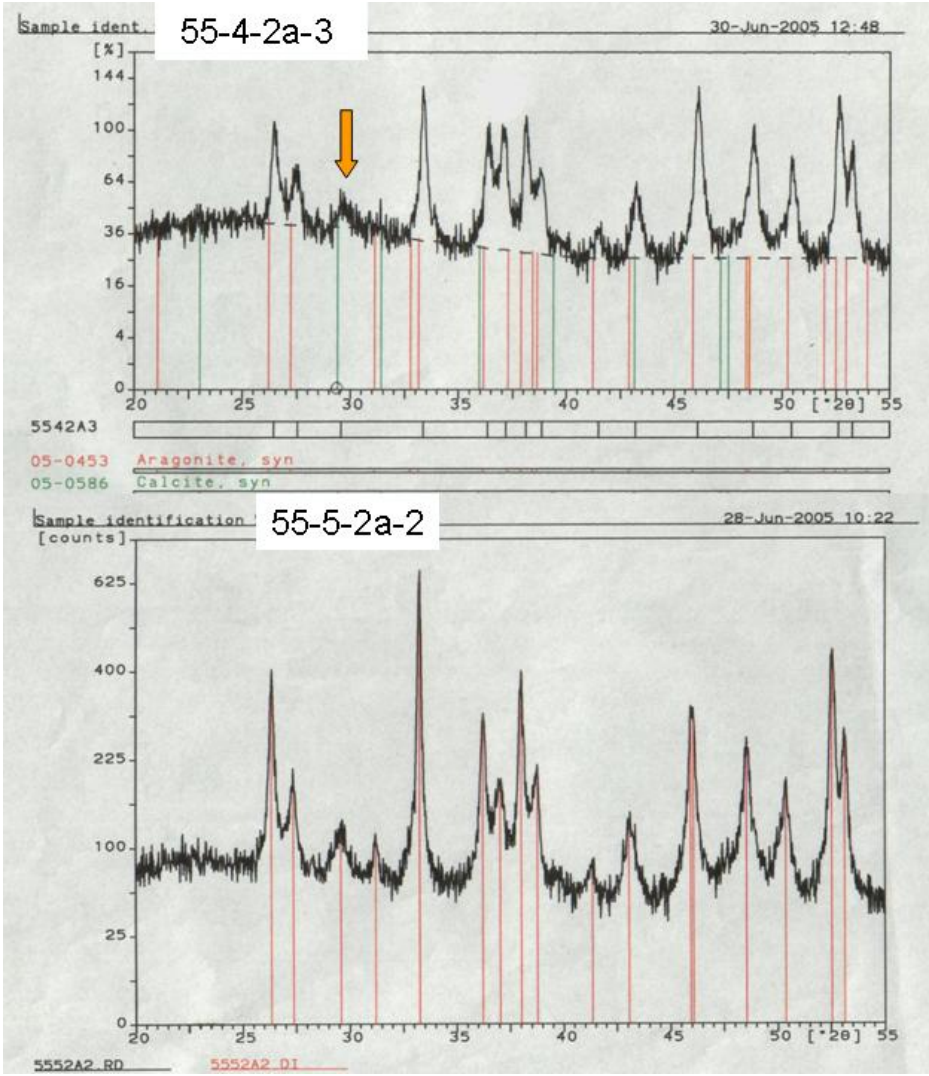

Figure 21b 1. Results of X-ray diffraction-XRD analysis for internal parts of the shells (samples PAN-2004-55-5-2a-2 and PAN-2004-55-4-2a-3). Red lines represent peaks of aragonite. Green lines represent peaks of calcite. Dashed lines represent minimal umbral to consider significant values. Orange arrow shows significant peak of calcite in shell 55-4-2a-3.

4.5.4 Preliminary oxygen and carbon isotope analysis with MS:

Analysis from the external part of selected shells (tubes PAN-04-55-1: 8 sub-samples, PAN-04-55-2: 14 sub-samples, PAN-04-55-3: 12 sub-samples) was performed to identify the best way for sub-sampling the shells for the subsequent oxygen and trace element analysis. Results of this showed the following:

1) There were detectable values for oxygen and carbon isotopes (Table 15). Therefore, it was possible to detect the isotopic composition and their variation from the external part of the shells.
2) All the shells showed peaks in the oxygen isotopic concentration approximately around 20mm from the tip of the tubes. Bearing in mind that the annual growth rate was 24mm (see section 9.5.1) and the date of collection was August 2004, these

peaks would correspond to the beginning of 2004 (Figure 22), the up-welling period in the area (potential negative relation with SST).

3) There was a strong and negative effect of the resin on the results. Samples of pure resin (see Table 15, port 39) were 17 times more negative for $\delta^{18}O$ than mollusc samples. In the same way, samples of molluscs with resin (Figure 22, see table 15, port 18) were at least twice more negative for $\delta^{18}O$ than samples without resin (see ports 28 to 32 in Table 15). It is essential to mention that samples were run from port 1 to 39 to prevent any effect of the resin (port 39) on the analysis. Samples of *E. tulipus* without resin showed similar values to samples of the mollusc *Tridacna* sp (see Annex 5, ports 9 to 18 for *Tridacna* sp with values for $\delta^{18}O$ between -0.738 and -3.563 PDB) that were run together in order to confirm performance (see http://www.geos.ed.ac.uk/research/globalchange/group4/Tridacna.html for more results of *Tridacna* sp).
4) The resin not only affected the results in the specific sample but it may cause a "memory effect" for subsequent samples as it was evident in port 18 for sample T1 (see Table 15) and the subsequent marble standards in ports 19 (value for $\delta^{18}O$: -3.033 PDB) and 20 (-2.664 PDB, mean value of $\delta^{18}O$ for MAB2B standard is -2.475PDB). Values in the next coral standards were again normal (value for $\delta^{18}O$ in port 21: -4.846, mean value of $\delta^{18}O$ for COR1D standard between December 2003 and March 2005 at Edinburgh University laboratories was -4.958). Therefore it was necessary to avoid the resin for the next analyses. Gordon (2002), in analysis for trace elements in otoliths of different species of fish, in which he tested the effect of resin in the analysis, found similar results: lower measurable concentrations of elements than in samples without resin. He concluded from his results that when the resin is included in the sample the final sample dilution is higher than it should be, resulting in lower than expected element concentrations.
5) The range for samples without resin was between -2.728 and -1.341PDB ($\Delta\delta^{18}O$ aragonite -1.39) and the average -2.227PDB (Figure 22). However these values correspond to a very small part (1 year approximately) of the chronological range that was expected to be analyzed.
6) Samples clean of epifauna or resin (see Table 15, sample T10 or T12) seemed to be almost identical in isotopic composition to samples without resin and few epifauna (see Table 15, sample T11) or very similar to samples completely covered with epifauna (see Table 15, T8 and T9). Therefore, the results did not show an evident effect of epifauna on the analysis. However, it was crucial to avoid epifauna in the

sampling due to two basic factors: a) the epifauna will grow on the mollusc shell some time after its deposition, creating noise in the time scale, b) the potential difference in the composition in terms of proportions of calcite-aragonite of each type of shell, epifauna and mollusc, may create extra noise in the analysis.

7) Although the methodology established at Edinburgh University for MS from drilled samples suggests the use of between 0.1 and 0.3 mg of sample, it was recommended that almost the maximum (0.3 mg) should be taken in order to reduce noise by effect of contamination or size in the samples. The reason being that smaller amounts produce bigger dilutions during the analysis and may result in more negative values.

From the results of this preliminary analysis it was decided to carry out analysis from the internal part of the shell (to avoid epifauna) and cut the tube to access directly the internal bands. In this way it was not necessary to use resin (used originally to give strength to the shells for drilling) and therefore the resin effects were avoided.

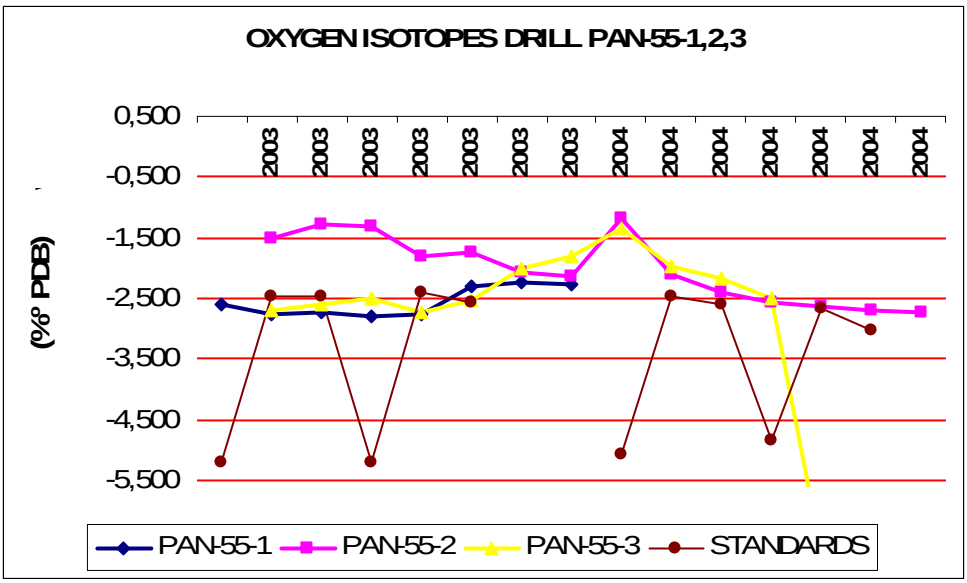

Figure 22. Preliminary oxygen isotope analysis for samples PAN-04-55-1 to PAN-04-55-3. Last value in PAN-04-55-3 (-6.700 PDB) corresponds to port 18 in Table 15, sample with resin. Standards with values between -4.5 and -5.5 correspond to coral standards (COR1D). Standards with values between -2.5 and -3.5 correspond to marble standards (MAB2B).

Table 15. Preliminary results for $\delta^{18}O$ and $\delta^{13}C$ isotopes analysis from sample PAN-04-55-3. *Mean value for MAB2B (analysis between December 2003 and March 2005 at Edinburgh University laboratories) is -2.475 PDB for $\delta^{18}O$ and 2.424 PDB for $d^{13}C$. ** Mean value for COR1D (analysis between December 2003 and March 2005 at Edinburgh University laboratories) is -4.958 PDB for $\delta^{18}O$ and -0.688 PDB for $d^{13}C$. For results of preliminary isotopes analysis with samples PAN-04-55-1 and PAN-04-55-2 see Annex 5. The port number corresponds to the position in the special container were the samples were deposited for the MS analysis. X/X?: presence of the item.

SHELL PAN-04-55-3							
PORT/DATE	**SAMPLE ID**	**µg SAMPLE**	**d13C (‰PDB)**	**d18O (‰PDB)**	**OBSERV.**	**RESIN**	**EPIFAUNA**
32(10-03-05)	T12	0,23	0,021	-2,683	Oldest band		
31(10-03-05)	T11	0,14	0,519	-2,605			X
30(10-03-05)	T10	0,16	0,385	-2,516			
29(10-03-05)	T9	0,10	0,138	-2,728			XX
28(10-03-05)	T8	0,13	0,381	-2,531			XX
27(10-03-05)	T7	0,19	-0,957	-2,008		X?	X
26(10-03-05)	T6	0,13	-1,116	-1,801		X?	X
25(10-03-05)	T5	0,23	-2,225	-1,341		X?	X
24(10-03-05)	T4	0,21	-2,511	-1,963		X?	
23(10-03-05)	T3	0,20	-1,711	-2,164		X?	
22(10-03-05)	T2	0,25	0,629	-2,514		X?	
18(10-03-05)	T1	0,15	-3,152	-6,700	Youngest band	X?	
STANDARDS PAN-04-55-3							
39(10-03-05)	**RESIN**	0,16	-34,941	-35,237			
38(10-03-05)	COR1D	0,170	-0,791	-5,212	Final standards		
37(10-03-05)	MAB2B	0,250	2,565	-2,416			
36(10-03-05)	MAB2B	0,100	2,354	-2,568			
35 TO 33 (10-03-05)	PAN-55-1						
32 TO 22 (10-03-05)	PAN-04-55-3						
21 (10-03-05)	COR1D	0,300	-0,539	-4,846	First standards		
20 (10-03-05)	MAB2B	0,240	2,290	-2,664			
19 (10-03-05)	MAB2B	0,250	1,922	-3,033			
18 (10-03-05)	PAN-04-55-3						
17 TO 4 (10-03-05)	PAN-04-55-2						
3 (10-03-05)	COR1D	0,20	-0,758	-5,143			
2 (10-03-05)	MAB2B	0,25	2,582	-2,276			
1 (10-03-05)	MAB2B	0,18	2,476	-2,443			

Results of Pearsons correlation analysis, performed to identify a potential correlation between carbon and oxygen isotopes values, showed “no correlation” between the two variables when performing the analysis for all the samples from the three shells (Figure 23). Correlation between the two isotopes would make the oxygen isotopes signal more

difficult to interpretate because that would implies a kinetic or biological effect of carbon isotope in the accumulation of oxygen isotope (Adkins *et al.* 2003). Therefore, the evidence of no correlation was a positive result for the whole target: the confirmation of *E. tulipus* as a potential proxy indicator of water mass conditions.

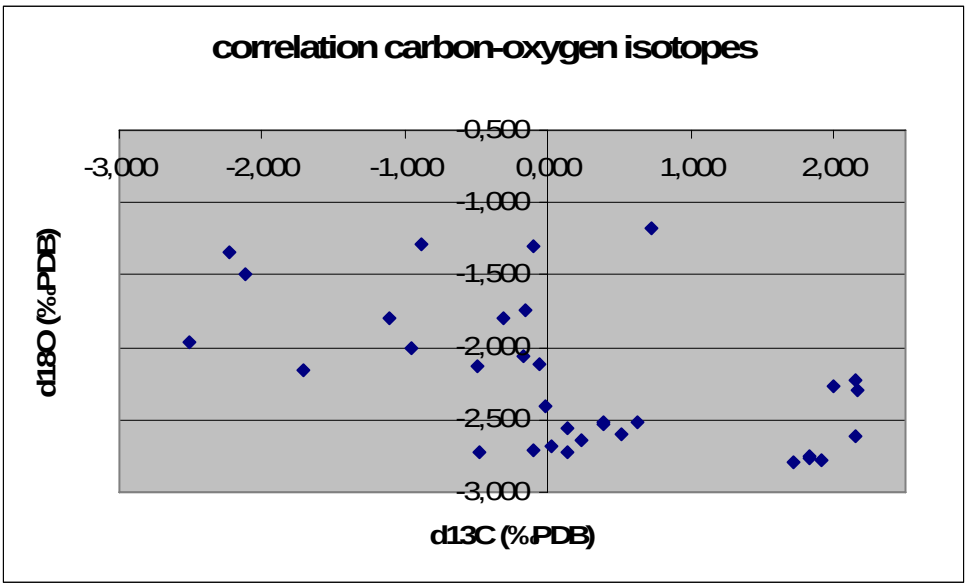

Figure 23. Correlation analysis between carbon and oxygen isotopes. Pearsons correlation coefficient for all the samples (PAN-04-55-1,2,3)= 0.081.

4.5.5. Oxygen isotopes in sea water:

The MS analysis for the four water samples showed the following results:

Sample A (north of Contadora Island): -0.26 δ^{18}O VSMOW

Sample B (North of San Telmo Island): -0.43 δ^{18}O VSMOW

Sample C (West Isla del Rey, Sucio River delta): -0.04 δ^{18}O VSMOW

Sample D (West of Chapera and Mogo Mogo Island): -0.54 δ^{18}O VSMOW

Average: -0.32 δ^{18}O VSMOW

Results of oxygen isotopes from the area reported by other authors at the Goddard Institute for Space Studies-NASA (Schmidt *et al.* 1999) show values between -0.5 and 0.5 VSMOW (Figure 24). The parameters used to access the information related to oxygen isotopic composition of the water mass in the area were: Latitude (-10 to 10), Longitude (-80 to -100). The database showed a total of 141 matches (values of δ^{18}O VSMOW) related to the following references: Wellington *et al.* (1996), Wantabe *et al.* (2001), Laube-L'Enfant (1996), Epstein and Mayeda (1953), Fairbanks *et al.* (1982), Benway and Mix (2004). Therefore, the results obtained with the 4 samples are in agreement with those reported previously for the area.

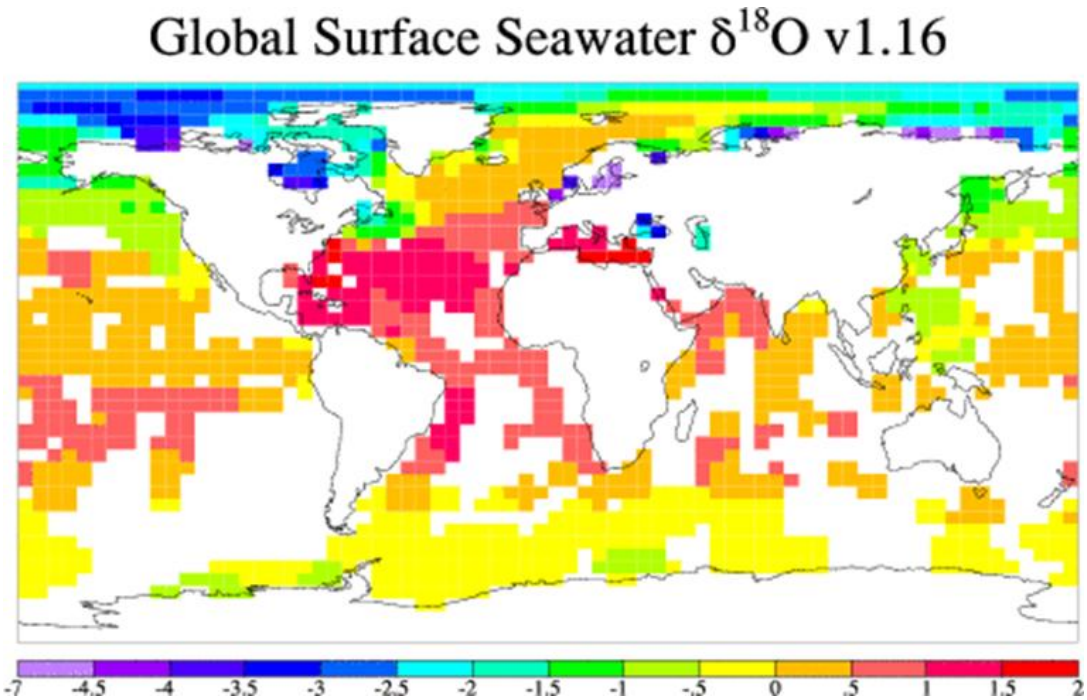

Figure 24. Global values of oxygen isotope composition of sea water. Modified from http://data.giss.nasa.gov/o18data/ (Schmidt *et al.* 1999).

4.5.6. Historical records of Sea Surface Temperature-SST and salinity:

Sea Surface Temperature-SST (Figure 25) and salinity records (Figure 26) collected at the Smithsonian Tropical Research Institute (STRI) show that:

1- There is an up-welling between January and April of each year that can be identified by a reduction in SST (Figure 25) and an increase in salinity (Figure 26). The lowest temperatures are in March and the highest between May and July of each year (Figure 25).

2- The historical mean temperature for the last 15 years (1992-2005) in Las Perlas Archipelago (see Figure 25) was 26.63°C, but temperatures as low as 14.7°C (Saboga, 22-02-2001) and as high as 30°C (Saboga, 9-11-1997) have been recorded. These values were used for comparisons with oxygen and trace element analysis.

3- The mean records of temperature for the last 17 years (1990-2005) in Panama Bay (see Figure 26) were 27.4°C for T°1 (morning) and 28.0 for T°2 (afternoon). These values were used only to assess the general behaviour of SST with respect to salinity records for Panama Bay. Although there was a difference of approximately 1°C between the records of temperature for Panama Bay and Las Perlas Archipelago, with lower values in the archipelago, both records showed the same pattern: reduction in sea water temperature between January and April of each year (see Figure 25 and 26).

4- The historical mean salinity for the last 17 years (1990-2005) in Panama Bay (see Figure 26) was 30.18 ‰, but the records showed salinities as low as 22‰ (21-10-1995) and as high as 44.8‰ (4-02-2005).

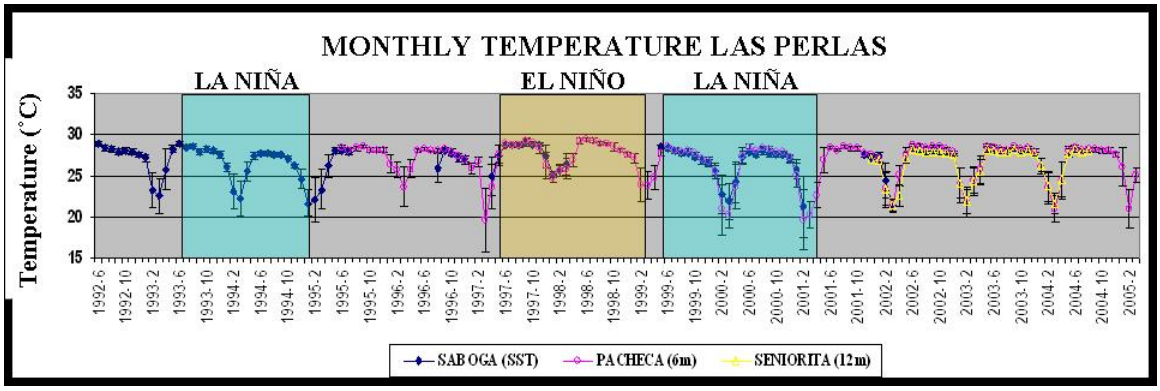

Figure 25. Monthly values of Sea Surface Temperature-SST for Las Perlas Archipelago during the last 15 years (1992-2005). Information for Saboga Island (SST, in blue) was recorded between 1992 and 2002. Information for Pacheca Island (6m depth, in pink) was recorded between 1995 and 2005. Information for Seniorita Island (12m depth, in yellow) was recorded between 2001 and 2004. Data sets were provided by CARICOMP and the Terrestrial-Environmental Sciences Program of the Smithsonian Tropical Research Institute.-STRI (http://striweb.si.edu/esp/physical_monitoring/download_orts.htm).

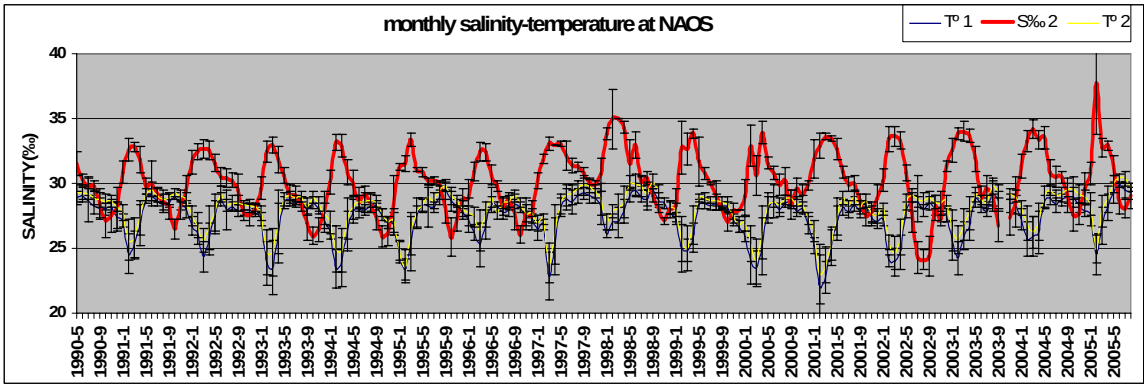

Figure 26. Monthly Salinity (in red) and temperature (blue and yellow) during the last 17 years (1990-2005) from Panama Bay. Data sets were provided by the Smithsonian Tropical Research Institute.-STRI (www.stri.org).

4.5.7 Trace element analysis with ICP-OES:

A total of 36 drilled samples from the selected shell (PAN-04-55-8) were analyzed with this technique: 5 samples from 2004, 7 samples from 2003, and 6 samples for each year between 1999 and 2003.

Results of analysis for Mg and Sr are shown in Annex 6. Mg/Ca ratios showed values between 0.3184 and 1.2397ppm (maximum SD: 0.6888ppm), while Sr/Ca ratios showed values between 1.6921 and 2.4530ppm (maximum SD: 0.3287ppm). There were no results for Ba because all the measurements for Ba were under the instrumental Limits of Detection-LOD (very low concentrations) and, therefore, were set to zero by the equipment. The explanation for this, according to the personal communication expertise

at SAMS, is that the few Ba atoms present in the samples were attached to the glass tubes used to read the samples in the spectrometer due to the fact that the samples were set in the tubes the previous night and storaged for 10 hours before the analysis. Therefore, the elements had all night to attach to the glass surface before the reading but this process was more obvious in Ba because of its low concentrations.

Before the comparisons between the elements (Mg, Sr) and the SST records, and due to the fact that the variables "weight, Mg/Ca and Sr/Ca" were shown not to be following a normal distribution (see results in Figure 27), which is expected for biological data and especially for these annual series, the Kendall rank correlation coefficient-T (non parametric) was performed to see any potential correlations between the amount of sample used for the test (weight) and each of the elements (Mg/Ca and Sr/Ca ratios).

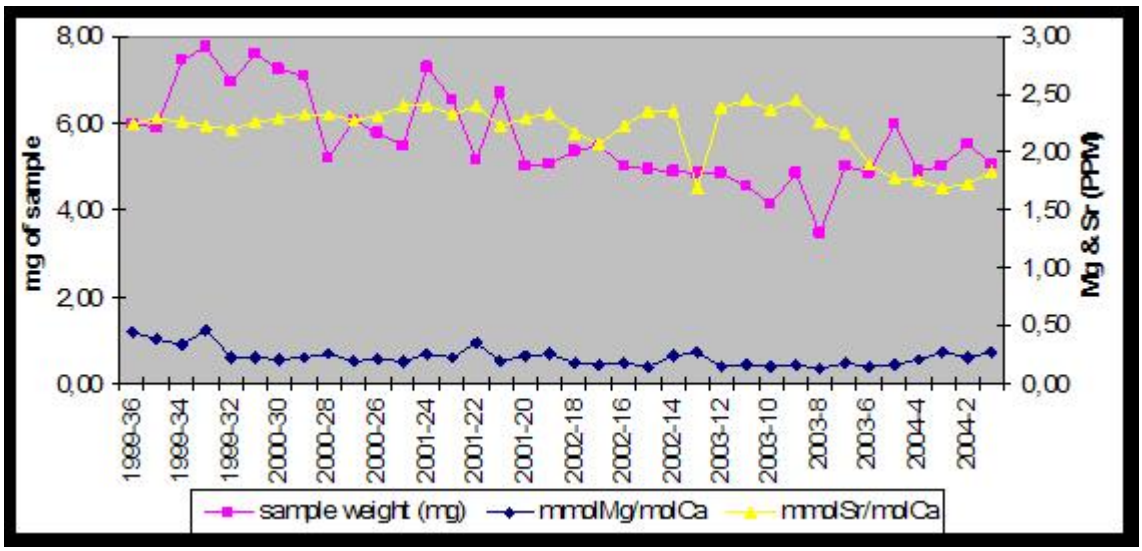

Figure 27. Results of ICP-OES analysis for Mg/Ca and Sr/Ca ratios in ppm (Mmol/mol) and their relation to the amount of sample used in the analysis (mg). X axis represents the time series as year-number of drilled sample. Test of normality (kolmogorov-Smirnov) for weight (Mean= 5.626, St. Dev= 1.044, N=36 , KS= 0.165, P.value= 0.018 < α=0.05) showed that this variable was not following a normal distribution. Test of normality (kolmogorov-Smirnov) for Mg/Ca ratios (Mean= 0.5995, St. Dev= 0.2169, N=36 , KS= 0.163, P.value= 0.023 < α=0.05) and for Sr/Ca ratios (Mean= 2.194, St. Dev= 0.2301, N=36 , KS= 0.251, P.value= <0.010 < α=0.05) showed that these variables were not following a normal distribution. Kendall rank correlation coefficient-T (non parametric) showed correlations between sample weight and Mg/Ca ratios but not between sample weight and Sr/Ca ratios.

The Kendall coefficient-T showed that the variation in the Mg/Ca ratios are highly related to the amount of sample (mg) used for the analysis (K1=0.3444, Z1=2.9557, P1=0.0016/ Z1>P1=highly significant; See Annex 7). However, there was no correlation between Sr/Ca ratios and the amount of sample used for the analysis (K2=-0.0508, Z2=-0.4359, P2>0.5, Z2<P2=no correlation; see Annex 7).

Due to the fact that both elements, Mg and Sr, are components of each sample, the results of correlation with only Mg represent an undercover noise in the results for Mg,

probably a trend in the series. Chatfield (1975) mentions that discrete time series, like the data obtained here, may include trends or long term changes in the mean as part of their variation, which must be eliminated before any time series analysis, especially under the ARIMA model. Indeed, results of analysis of trace elements for other molluscs have shown trends in the results for Mg/Ca ratios (http://www.geos.ed.ac.uk/research/globalchange/group4/Tridacna.html, Elliot M., Com. Pers.). To eliminate the trend from the results for Mg/Ca ratios was very difficult in this case because of the way in which samples were collected (overlapping of one sample with the previous one, see methodology ICP-OES, page 93). Therefore, and in order to see general patterns between Mg/Ca ratios and SST, a regression analysis was performed on the original data (Mg/Ca vs. SST, Sr/Ca vs. SST). The decision for the regression analysis and its analysis of variance was based on the facts that (Steel and Torrie 1988):

- The assumption of normality is not necessary in order to estimate the components of the variance
- Even the most recommended transformation for fractions like the values obtained from the Mg/Ca and Sr/Ca ratios (arcsinχ transformation) is not completely recommended because there is change in the variance and standard deviation of each variable (Figure 28).

Figure 28 shows that the assumptions established during the analysis (negative relation for Sr/Ca vs. SST and positive relation between Mg/Ca ratios and SST, see 4.4.3 statistical analysis for trace elements with ICP-OES, page 104) were not evident in the graphics. Only in the case of Mg (Figure 28 top), increases in SST during the first part of each year were followed by increases in Mg/Ca ratios, except during 2003 and 2004, where the signal was more confused. The negative relation between Sr and SST was not evident from the praphic (Figure 28 bottom).

The linear regression analysis proved that Mg does not behave according to SST (Figure 29). In the figure is evident that there was almost no slope and, therefore, there was no relation, and “a” (intercept on the “y” axis when X=0) was never zero. In the same way, the 95% confidence bands and 95% prediction bands showed that there was a lot of information that was outside the equation. The above means that the equation could not predict the relation between Mg/Ca ratios and SST in Pacheca Island. The “no relation” obtained between Mg/Ca and SST could be related with the noise in the Mg/Ca ratios (the overlapping of annual bands within samples and the trend found from the Kendall

rank correlation coefficient-T test between Mg/Ca and sample weight). This noise creates other layers of information, making the relation between Mg/Ca ratios and SST very difficult to predict.

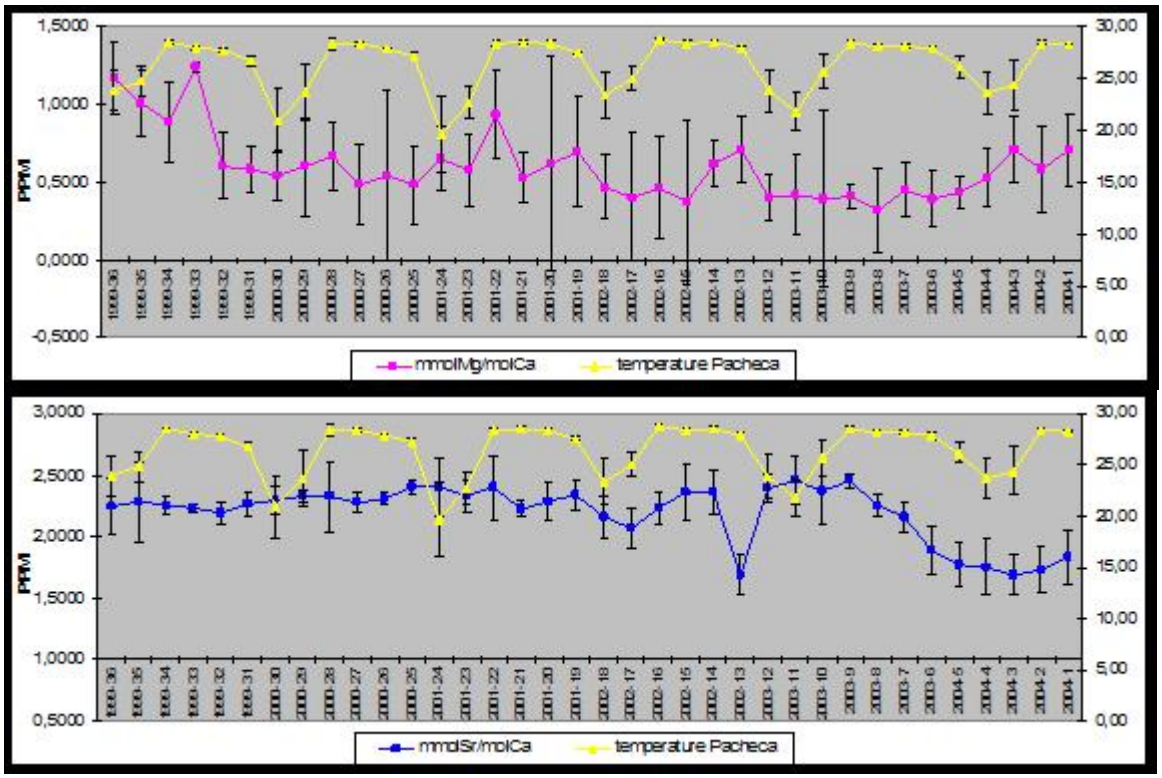

Figure 28. Potential relation between each element (ratios Mg/Ca, Sr/Ca in ppm) and the temperature for the same period. Top shows Mg/Ca ratios (± Stand. Dev) and values of SST (± SD) every two months in Pacheca Island (Las Perlas Archipelago). Bottom shows the same relation for Sr/Ca ratios and SST in Pacheca Island. X axis represents the time series as year - number of sample. SST Data sets were provided by the Terrestrial-Environmental Sciences Program of the Smithsonian Tropical Research Institute.-STRI (http://striweb.si.edu/esp/physical_monitoring/download_orts.htm).

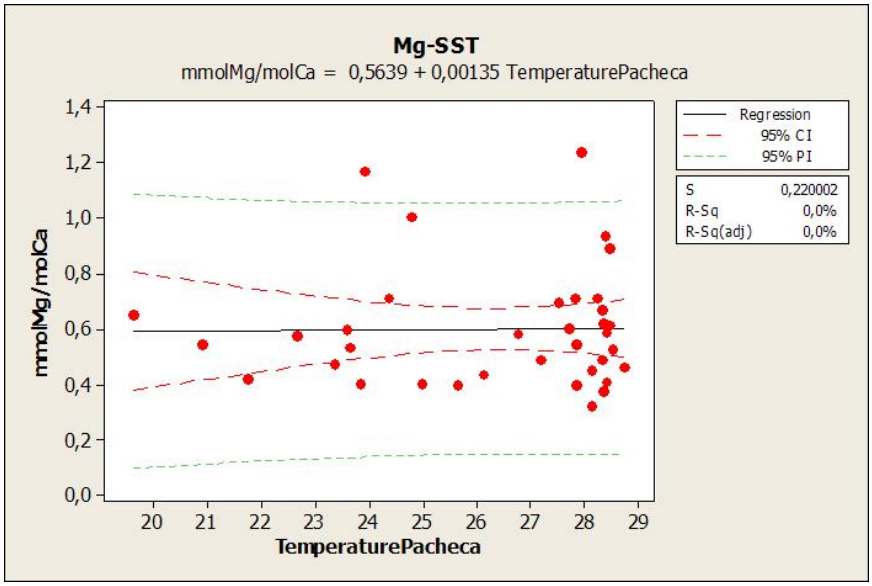

Figure 29. Linear regression analysis between Mg/Ca ratios and values of SST every two months in Pacheca Island (Las Perlas Archipelago). The analysis of variance of the regression showed a very low variance ratio (F=0.01) and a high value of P (0.927) that means that the null hypothesis (no relation between variables) must be accepted. SST Data sets were provided by the Terrestrial-Environmental Sciences Program of the Smithsonian Tropical Research Institute.-STRI (http://striweb.si.edu/esp/physical_monitoring/download_orts.htm).

In the case of the second element, Sr/Ca, the linear regression analysis showed that Sr does not behave either according to SST (Figure 30). In the figure is evident that there is a very small negative slope. The confidence bands and prediction bands also show that there was a lot of information outside the equation. The "no relation" obtained in this case maybe be due to the noise during the collection of the samples (the overlapping of annual bands within samples). This noise creates other layer of information, making the relation between Sr/Ca ratios and SST confused and difficult to predict.

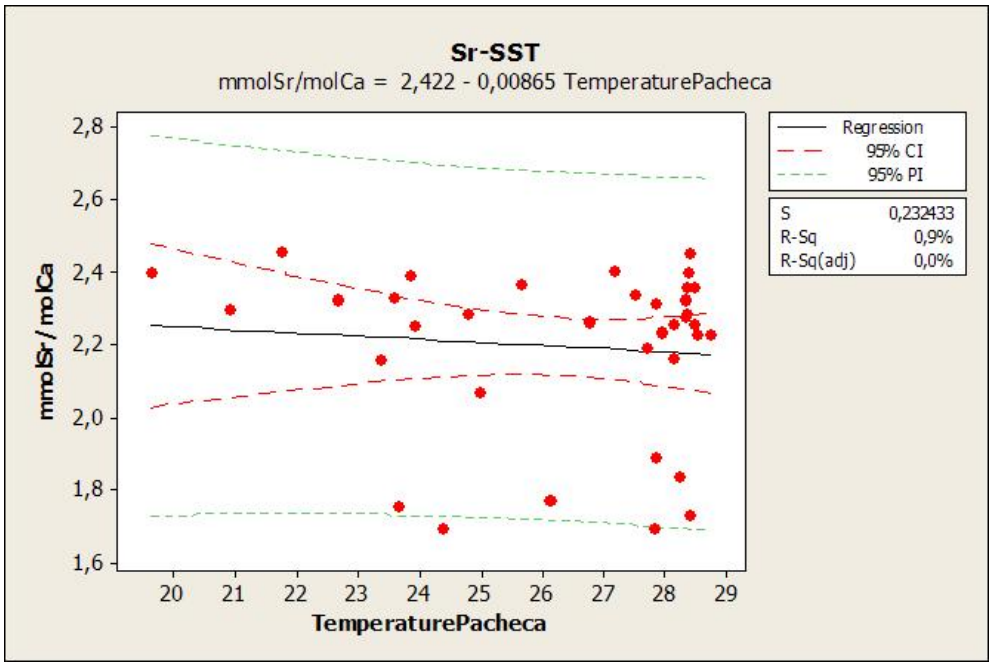

Figure 30. Linear regression analysis between Sr/Ca ratios and values of SST every two months in Pacheca Island (Las Perlas Archipelago). The analysis of variance of the regression showed a low variance ratio (F=0.31) and a value of P higher than 0.5 (P=0.580). The above means the equation did not have enough statistical significance and, therefore, the null hypothesis of no relation between variables needed to be accepted. SST Data sets were provided by the Terrestrial-Environmental Sciences Program of the Smithsonian Tropical Research Institute.-STRI (http://striweb.si.edu/esp/physical_monitoring/download_orts.htm).

4.5.8 Trace element analysis with LA-ICP-MS:

A total of 29 ablations were collected directly from a transverse section of the selected shell (PAN-04-55-8) and analyzed with LA-ICP-MS (Figure 31). Results of the analysis for Mg/Ca, Sr/Ca and Ba/Ca ratios in ppm (±SD) are shown in Figure 32. The figure show the results in chronological order, from sub-sample 22 to sample 1, including the second line of spots (sub-sample 29 to 23) in between the first line.

Mg/Ca ratios showed values between 7.05 and 49.42 ppm (Mg25: 7.05 - 30.38 ppm, Mg26: 9.64 - 49.42 ppm), with Standard deviation that reached in some cases 30 ppm, which is similar to the maximum concentration (Figure 32a). Large standard deviations were expected since the performance for Mg was not very good during the analysis but it was essential to keep the established conditions to avoid compromising the results of the other elements. Another important point is that in this general plot of data the linear rising trend in the concentrations of Mg from the oldest (sub-sample 22) to the youngest bands (sub-sample 1, Figure 32a) was more evident. The trend was overlapping some fluctuation or seasonality. The basic difference between the two ratios was the slope (slightly higher in Mg26/Ca ratios) and the intercept on the y axis that was slightly higher in Mg26/Ca ratios (Figure 32a) showing that the lines were almost parallel. For the purposes of the comparisons with SST, only Mg25 was used.

Sr showed the highest concentrations, with ranges between 63.10 and 99.20 ppm for Sr84/Ca, and smaller standard deviations that reached in some cases 17 ppm (Figure 32b). Although the concentrations of Sr86/Ca showed the same pattern (Figure 32b) the values were very high (ranges between 1170.34 – 1836.88 ppm and standard deviations that reached 18ppm). The high values in Sr86/Ca and their similar standard deviations to Sr84 suggested that Sr84/Ca was showing closer values to reality. Therefore, only Sr84 was used for the comparisons with SST. The general plot of the data for Sr84 also showed that there was a small, lineal and negative trend in the concentrations that was overlapping some fluctuation or seasonality.

On the other hand, Ba showed the lowest concentrations, with ranges between 2.62 and 17.58 ppm for Ba137/Ca, and big standard deviations that reached in some cases 29 ppm (Figure 32c), which is higher than the largest concentration obtained (17.58ppm).

Ba138/Ca ratios showed the same pattern as Ba137/Ca but the values were high (ranged between 16.89 – 113.32 ppm and standard deviations that reached 28 ppm). In the same way than with Sr ratios, the higher values in Ba138/Ca and their similar standard deviations in comparison with Ba137 suggested that Ba137/Ca was showing closer values to reality. Here again, only Ba137 was used for the comparisons with SST. The general plot of the data for Ba137 also showed that there was a linear rising trend in the concentrations that was overlapping some seasonality (values showed the effect of wet and dry seasons on the accumulation of the element) .

Fallon *et al.* (2003) in analysis of Mg/Ca and Sr/Ca ratios with LA-ICP-MS for corals also found higher Sr concentrations than Mg.

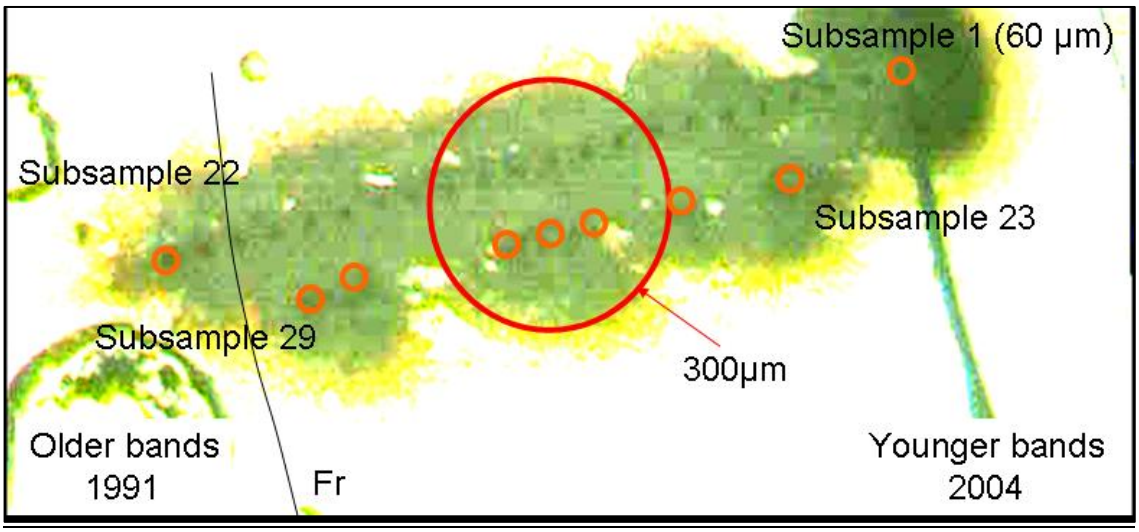

Figure 31. Analysis of trace elements from the selected shell (PAN-04-55-8) with LA-ICP-MS. Figure shows track of laser ablation spots on the transverse section. 300 µm correspond to the pre-ablation area. Size of each spot: 60 µm plus the aureola. Total size: 75 µm. Fr: fracture that correspond to the end of internal bands according to Figure 20. Younger bands 2004: area that corresponds to the internal part of the transverse section in Figure 20. Older bands 1991: area that corresponds to the end of internal bands and beginning of bioerosion (big hole marks in left part of this figure) in Figure 20.

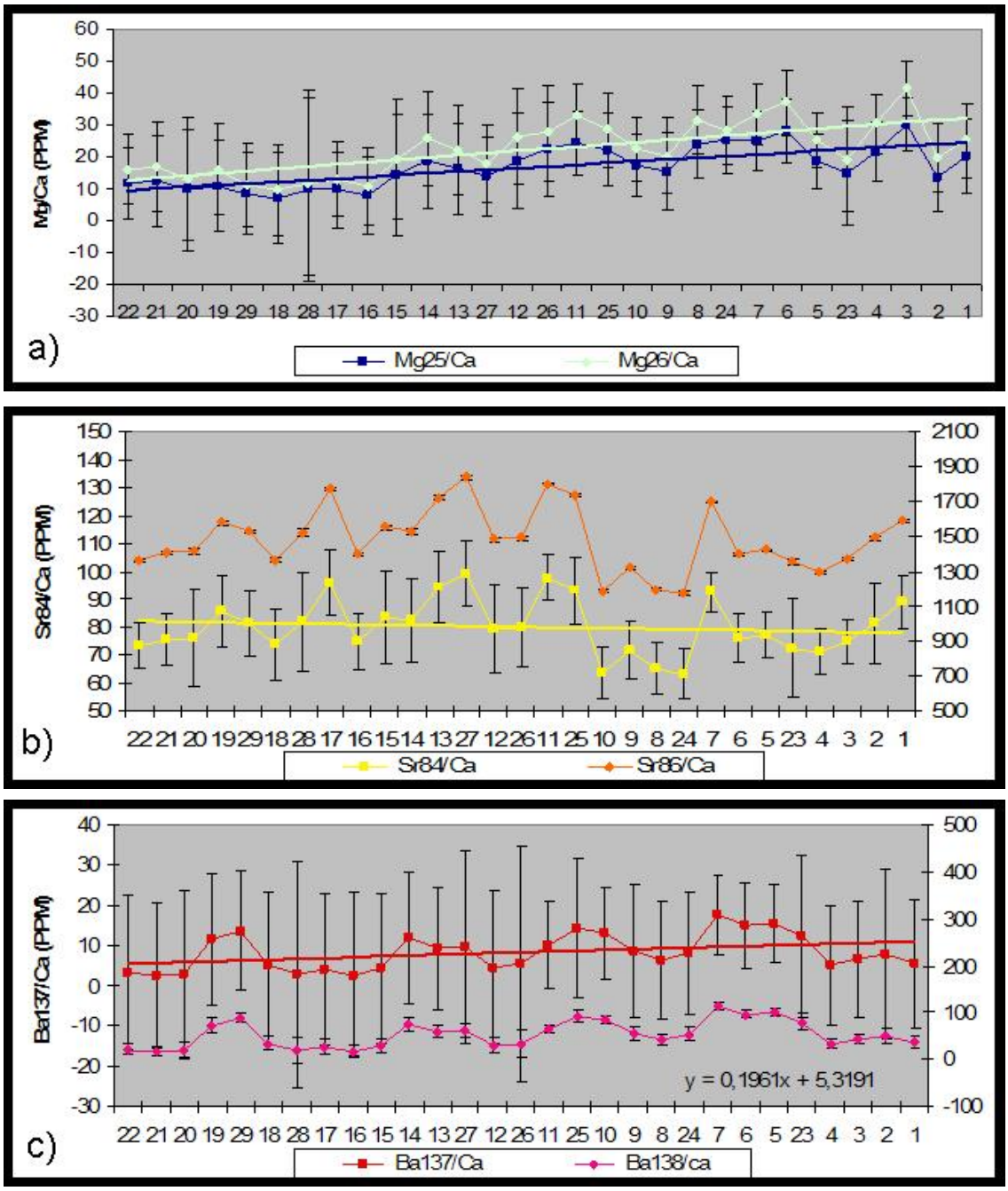

Figure 32. Results of trace elements' analysis (± SD) with LA-ICP-MS and estimated trend lines for samples collected from the selected shell (PAN-04-55-8). a): Mg/Ca ratios. b): Sr/Ca ratios. c): Ba/Ca ratios. X axis represents the number of the ablated sample.

The residuals (used to eliminate the trends for the comparison of Mg and Sr with SST, and Ba with salinity) were calculated from the linear equation obtained for the trend (fitting the linear function) as follow:

Y (residual)= (BX+A) –X

The new values were the mirror image of the originals (Figure 33).

For the comparisons, and in order to obtain equal periods of time from the ratios, only sub-samples from the first line of spots (sub-samples 1 to 22) were used.

Based on the results of the growth band analysis (see Figure 20: 14 years of total information from selected shell PAN-04-55-8, with last value on August 2004) and the fingerprint of the LA-ICP-MS on the sample that included all the bands (see Figure 31) it was estimated that the first 20 sub-samples would correspond to the 14 year period. The last two sub-samples (21 and 22) would be therefore part of the end of 1990 and beginning of 1991. Because the SST records at STRI began in June 1992, only sub-samples 1 to 20 were used for the comparisons. However, salinity records began in 1990 and therefore sub-samples 1 to 22 were used for the comparisons between Ba and salinity. From the SST and salinity monthly records, values every four months were selected, beginning in August 2004 backwards, for the regression analysis.

The results of the descriptive comparisons with the residuals (Figures 34 and 35) showed that:

-All the peaks in the residual values of Mg/Ca ratios were related to low values of SST (Figure 34a). Bearing in mind that the residuals were mirror images of the original data, a positive relation could be expected from the regression analysis.

-Reductions in the residual values of Sr/Ca corresponded, with some delay, to reductions in monthly values of SST (Figure 34b). Therefore, a negative relation (because residuals were mirror images of originals) should be expected.

- Reductions in the residual values of Ba/Ca ratios corresponded with reductions in monthly values of salinity (Figure 34c). Therefore, a negative relation (because residuals were mirror images of originals) should be expected.

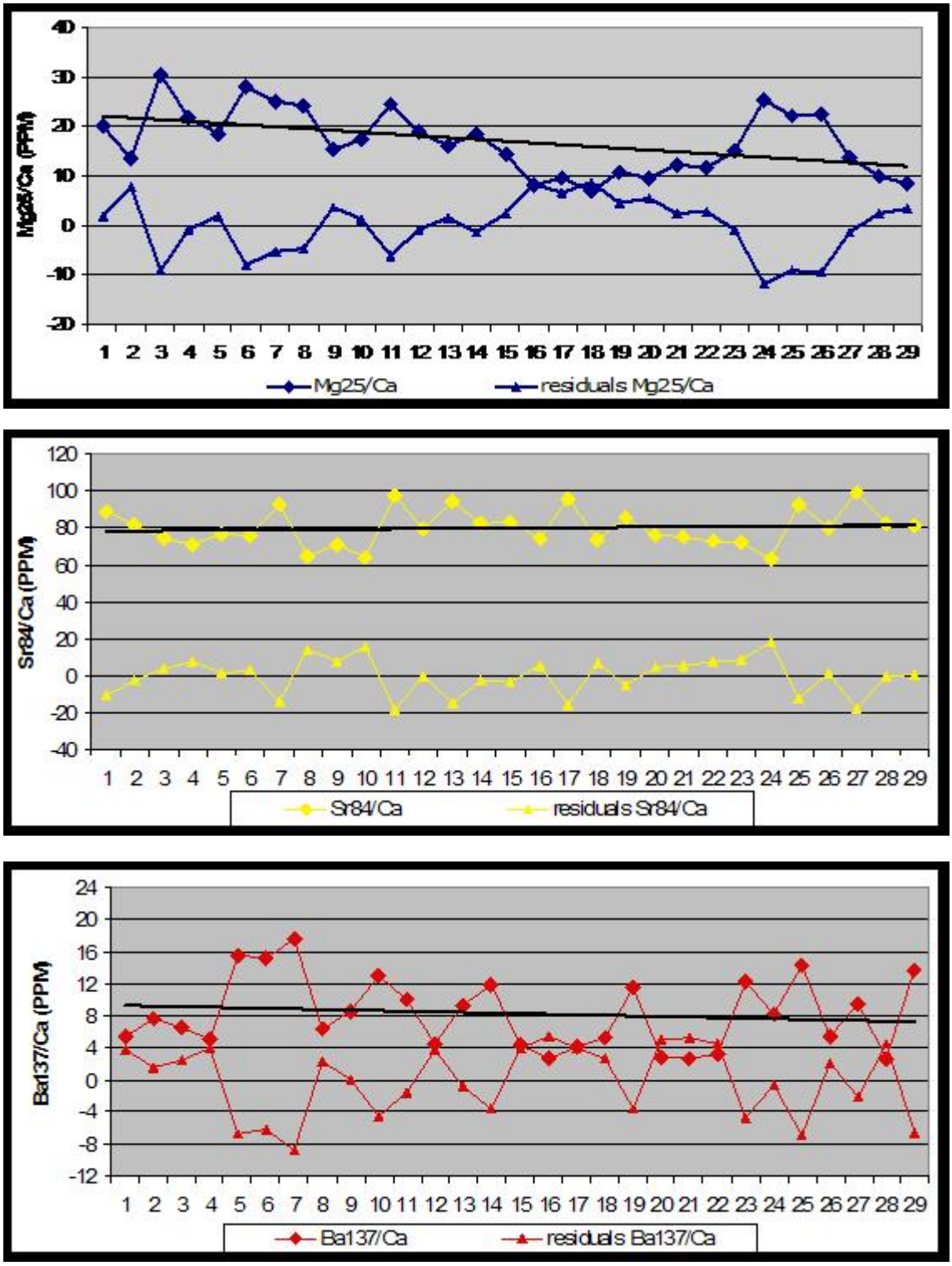

Figure 33. Results of fitting a linear function on Mg25/Ca, Sr84/Ca and Ba137/Ca ratios. The figure shows the original series, the linear trends and the new values or residuals.

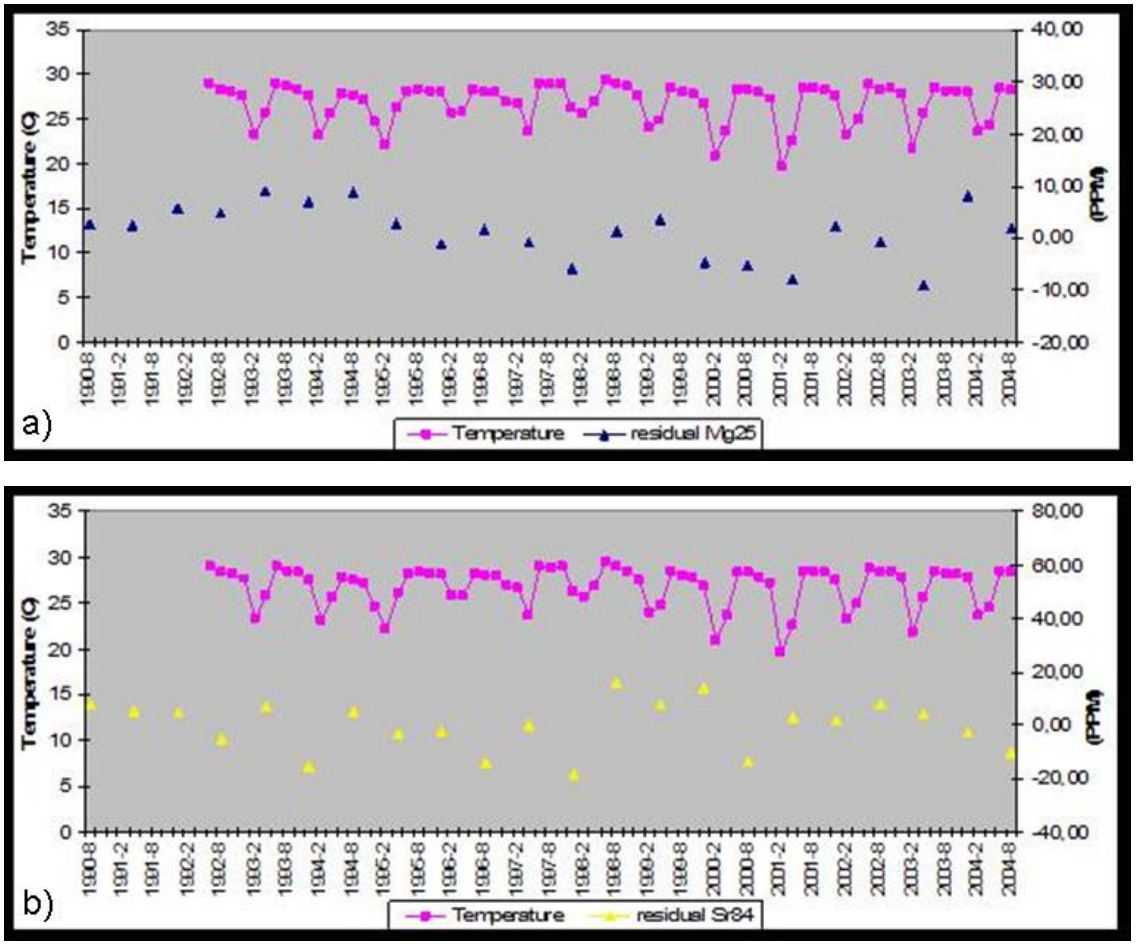

Figure 34. Potential relation between residuals of a) Mg25/Ca ratios and b) Sr84/Ca ratios with SST between June 1992 and August 2004. SST Data sets were provided by the Terrestrial-Environmental Sciences Program of the Smithsonian Tropical Research Institute.-STRI (http://striweb.si.edu/esp/physical_monitoring/download_orts.htm).

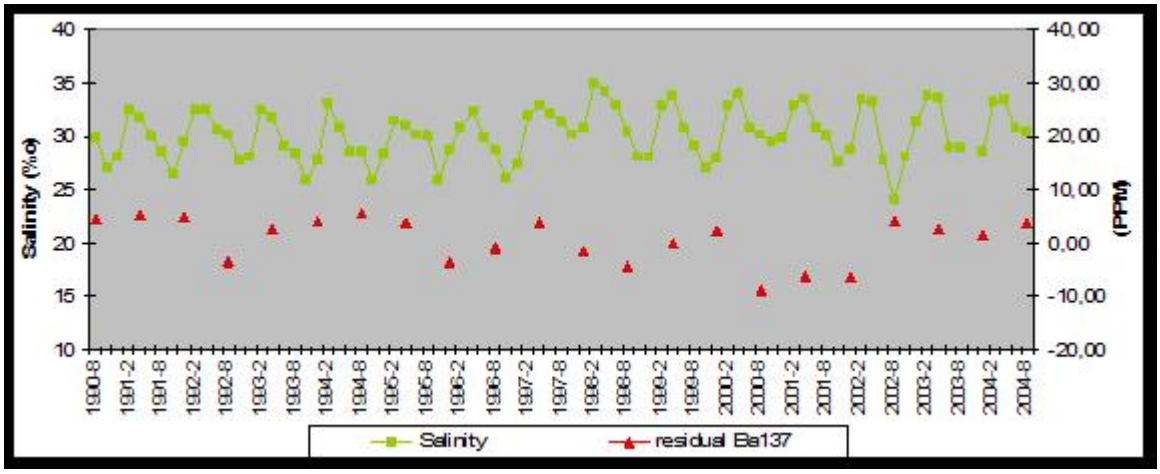

Figure 35. Potential relation between residuals of Ba137/Ca ratios and salinity between August 1990 and August 2004. Salinity data was provided by the Smithsonian Tropical Research Institute.-STRI (www.stri.org).

Results of the regression analysis proved that the residuals of Mg25/Ca behave in the same way as SST, which confirm the assumptions of positive relation between the variables (Figure 36). Indeed, the analysis of variance of the regression (Table 16) showed a higher variance ratio (F=1.93) than in the ICP-OES for Mg, and a probability lower than 0.5 (P=0.183). The above means that the linear regression had enough statistical significance to confirm the positive relation between Mg/Ca and SST.

Although part of the data was outside of the 95% confidence bands, all the information was included in the 95% prediction intervals. The above means that the prediction of the relation between variables (the equation) had better accuracy than the ICP-OES analysis for Mg (see Figure 29).

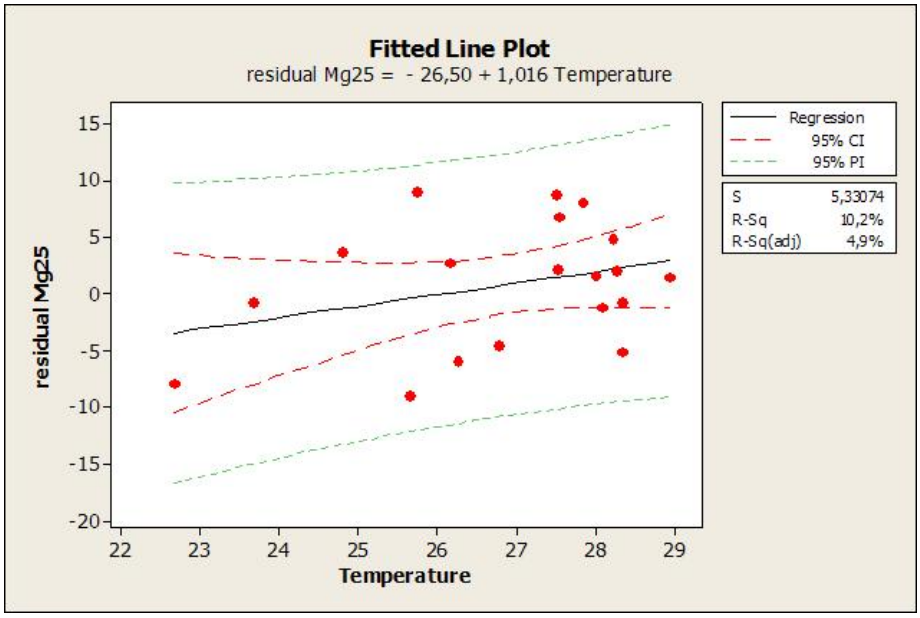

Figure 36. Linear regression analysis between residuals of Mg25/Ca ratios and values of SST every four months in Las Perlas Archipelago. SST Data sets were provided by the Terrestrial-Environmental Sciences Program of the Smithsonian Tropical Research Institute.-STRI (http://striweb.si.edu/esp/physical_monitoring/download_orts.htm).

Table 16. Analysis of variance for the linear regression analysis between residuals of Mg25/Ca ratios and SST from Las Perlas Archipelago.

Analysis of variance of regression					
SOURCE	DF	SS	MS	F	P
REGRESSION	1	54.889	548.886	1.93	0.183
ERROR	17	483.085	284.168		
TOTAL	18	537.974			

In the case of Sr, the regression analysis showed that the residuals of Sr84/Ca increased with reductions in SST (Figure 37) and, therefore, the assumptions of negative relation

between the variables were confirmed. The analysis of variance of the regression (Table 17) showed a higher variance ratio (F=0.56) than in the ICP-OES for Sr, and a probability lower than 0.5 (P=0.463). The above means that the linear regression was statisticaly significant to confirm the negative relation between Sr/Ca and SST.

Here again, part of the data was outside of the 95% confidence bands, but all the information was included in the 95% prediction intervals. Therefore, the prediction of the relation between variables (the equation) had better accuracy than the ICP-OES analyses for Sr (see Figure 30).

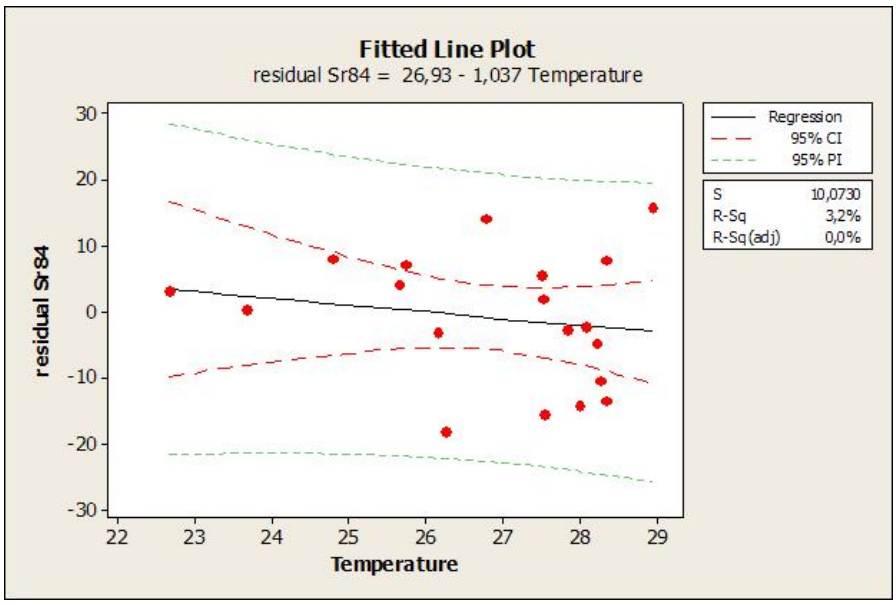

Figure 37. Linear regression analysis between residuals of Sr84/Ca ratios and values of SST every four months in Las Perlas Archipelago. SST Data sets were provided by the Terrestrial-Environmental Sciences Program of the Smithsonian Tropical Research Institute.-STRI (http://striweb.si.edu/esp/physical_monitoring/download_orts.htm).

Table 17. Analysis of variance for the linear regression analysis between residuals of Sr84/Ca ratios and SST from Las Perlas Archipelago.

Analysis of variance of regression					
SOURCE	DF	SS	MS	F	P
REGRESSION	1	57.21	57.208	0.56	0.463
ERROR	17	1724.93	101.466		
TOTAL	18	1782.13			

On the other hand, the regression analysis for the residuals of Ba137/Ca and salinity did not have enough statistical significance to confirm the equation of negative relation observed between the variables during the analysis (Figure 38). The low significance

was evident in the figure because most of the data was outside of the 95% confidence bands (confidence band smaller than expected) and one value was outside of the 95% prediction intervals. The "no relation" obtained in this case may be due to the high standard deviations observed during the analysis (see Figure 32c). This noise made the relation between Ba and salinity confuse and difficult to predict.

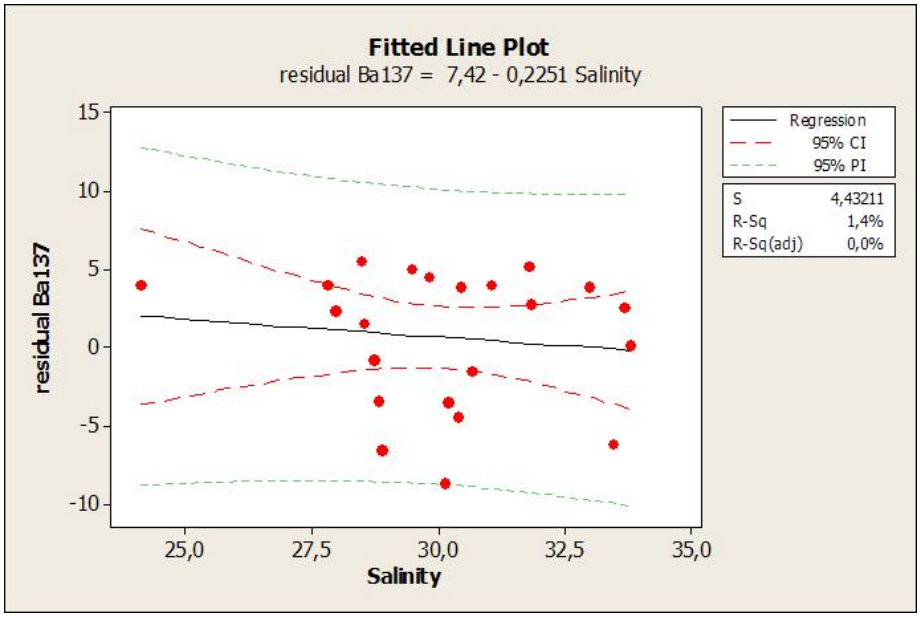

Figure 38 Linear regression analysis between residuals of Ba137/Ca ratios and values of salinity every four months in Panama Bay. The analysis of variance of the regression showed a low variance ratio (F=0.28) and a probability higher than 0.5 (P=0.601), which made the equation of negative relation non significant and, therefore, the null hypothesis (no relation between variables) needed to be accepted. Salinity data was provided by the Smithsonian Tropical Research Institute.-STRI (www.stri.org).

4.5.9 Trace element analysis with Ion Microprobe-IMF:

A total of 354 values for each element and each of the two transects were collected directly from a transverse section of the selected shell (PAN-04-55-8) and analyzed with this technique. From the total, 269 values in the first transect (T1) and 281 values in the second transect (T2), were between the fractures (Fr) observed in the internal layers (see Figure 20 bottom, page 112). Results of analysis for Mg/Ca, Sr/Ca and Ba/Ca ratios in ppm are shown in Figure 39.

Mg/Ca ratios (Figure 39 top) showed values between 124.78 and 739.51 ppm (Mg/Ca T1: 128.25 – 552.59 ppm, Mg/Ca T2: 124.78 – 739.51 ppm), ignoring the noise obtained around values 20 and 300 because of the fractures in the shell (see Figure 20 bottom, page 112). An important point is that in this general plot of data the linearly rising trend in the concentrations of Mg from the oldest (value No. 354 in T2) to the

youngest bands (value No. 2 in T2) was evident (Figure 39 top). The figure shows a negative linear trend because the data were organized in numerical instead of chronological order for the purposes of obtaining the linear equation to eliminate the trend and obtain the residuals for the comparisons with SST. It was also evident that the trend was overlapping some seasonality (effect of wet and dry seasons in the area on the accumulation of the element). The overlapping between the two tests (T1 and T2) for Mg and the other two elements was almost identical (see Figure 39 top, middle and bottom), which proved the reproducibility of the procedure. For the purposes of the comparisons with SST, only the second test was used because this one includes data until 2004.

Sr showed again, as in the LA-ICP-MS analysis, the highest concentrations (Figure 39 middle), with values between 1433.32 and 3578.53 ppm (Sr/Ca T1: 1433.32- 3578.53 ppm, Sr/Ca T2: 1493.59 – 3501.16 ppm), ignoring the noise around 0-20 and 300 because of the fractures in the shell (see Figure 20 bottom). In the plot a linearly rising trend in the concentrations of Sr from the oldest (value No. 354 in T2) to the youngest bands (value No. 2 in T2) was also evident. The figure shows again a negative linear trend because the data were organized in numerical instead of chronological order for the purposes of obtaining the linear equation to eliminate the trend and obtain the residuals for the comparisons with SST. As in the previous element, some seasonality was also evident but with peaks that corresponded with the lower fluctuations in Mg. For the purposes of the comparisons with SST and the other elements, only the second test (T2, data until 2004) was used.

On the other hand, Ba showed the lowest concentrations (as in the LA-ICP-MS analysis), with values between 13.58 and 40.06 ppm (Ba/Ca T1: 13.58 – 39.39 ppm, Ba/Ca T2: 15.05 – 40.06 ppm), ignoring the peaks higher than 42 ppm that corresponded to the fractures in the shell and “0” values that were registered as 40,000 counts/sec for the machine (see 4.4.2 laboratory analysis for trace elements with IMF, page 101). The peaks during the readings higher than 42 ppm were not due to contamination as was proved by the very low values of Si/Ca ratios (Figure 39 bottom). The difficulty in obtaining the Ba values was due to high values in the constant obtained from the OKA standard for corrections of the lectures for Ba and the very low levels of the element in the shell, near to the limits of detection of the equipment. For the

purposes of the comparisons with salinity and the other elements, only the second test (T2, data until 2004) was used.

From the set were eliminated the values that corresponded to the fractures (20 to 26 and 301 to 306) and "0" values or other noise (1-2 in Mg, 1-20 in Sr, and 1-2, 31-34, 148-149 in Ba). Based on figure 20, the first 300 values would correspond to information between August 2004 and the second fracture in 1991. The time period for SST records was from June 1992. Therefore, for comparisons of Mg and Sr with SST only the first 300 values of T2 were used. In the case of salinity, the records came from 1990 and, therefore, for comparisons with Ba were included values after the second fracture (306 to 354), especially because the values showed a similar pattern than those between the fractures and therefore could be part of the internal layers.

The residuals, for the comparisons with SST (Mg and Sr) and salinity (Ba), were calculated from the linear equation obtained for the trend (fitting the linear function) as follow:

$$Y\ (\text{residual}) = (BX+A) - X$$

The results of the descriptive comparisons of SST and salinity with the residuals (Figures 40 and 41) showed that:

-All the peaks in the residual values of Mg/Ca ratios were in general related to reductions in SST (Figure 40). Bearing in mind that the residuals were mirror images of the original data, a positive relation from the regression analysis could be expected.

-Although the values for Mg corresponded to a transformation from the original data there was still some rising trends in the residuals of Mg/Ca from the oldest (June 1992) to the youngest bands (August 2004).

-Reductions in the values for residuals of Sr/Ca were in general related to reductions in monthly values of SST (Figure 40). Therefore, a negative relation (because residuals were mirror images of originals) should be expected.

-There was a negative relationship between Mg and Sr because all the peaks in Sr corresponded with reductions in Mg and vice versa.

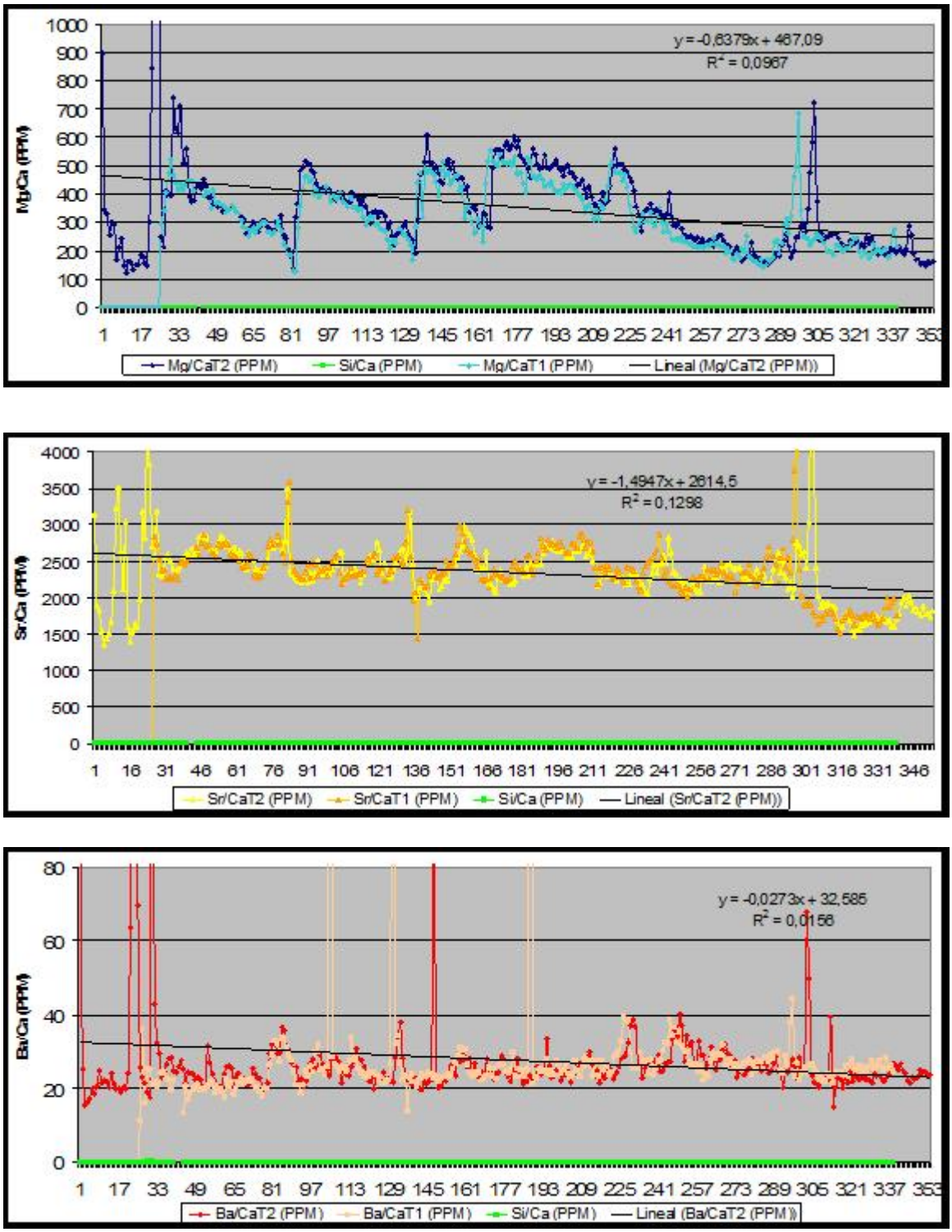

Figure 39. Results of trace elements analysis with IMF and estimated trend lines (fitting linear function) on Mg/Ca ratios (top), Sr/Ca ratios (medium), and Ba/Ca ratios (bottom) for samples collected from the selected shell (PAN-04-55-8).

-Based on the facts that the previous two elements show in some way the expected results and that the residuals are a mirror image of the original data, there should be a relationship between the peaks of Ba and peaks of salinity to obtain the expected negative relation. However, Figure 41 showed that reductions in the residual values of Ba/Ca ratios corresponded with peaks in monthly values of salinity. The above could be interpreted in two ways: 1) there was a potential positive relation (opposite to expectations), 2) there was a delay in the signal for Ba.

-There was still some rising trend in the residuals of Ba/Ca from the oldest (May 1990) to the youngest bands (August 2004) after the transformation (see Figure 41).

Results of the regression analysis proved a negative relationship between the residuals of Mg/Ca and SST (Figure 42), just opposite to the assumptions established during the laboratory before the statistical analysis. Indeed, the the analysis of variance of the regression (Table 18) showed a similar variance ratio ($F=1.88$) than in the LA-ICP-MS for Mg, and a probability lower than 0.5 ($P=0.172$). The above means that the linear regression had enough statistical significance ($P<0.5$), to confirm a negative relation between Mg/Ca and SST. These confusing (contrary to expected) results may be due to the trend that remained after the transformation.

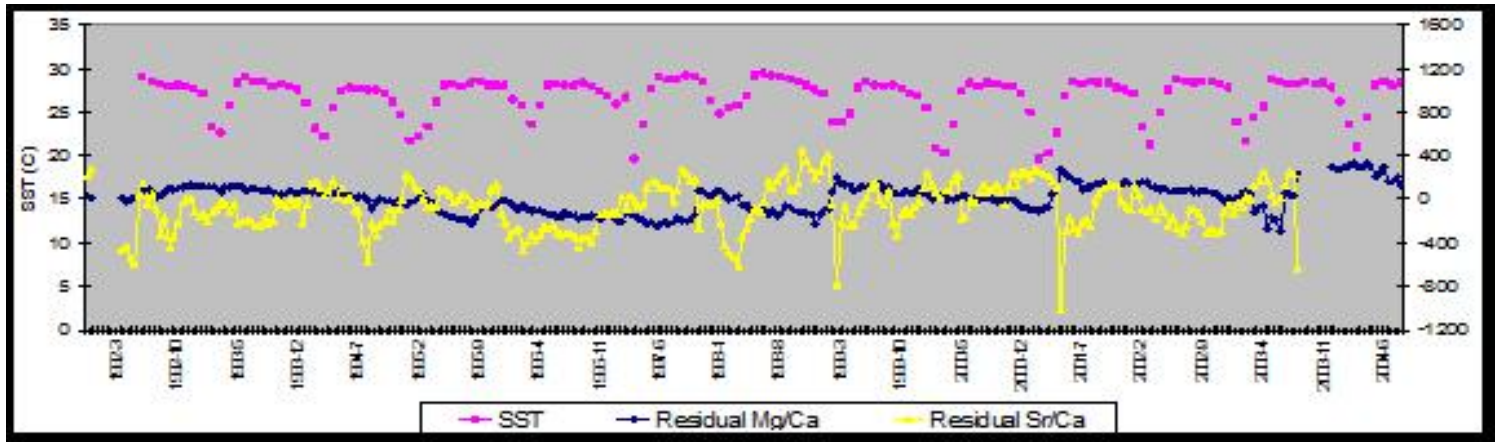

Figure 40. Potential relation between residuals of Mg/Ca ratios (blue) and Sr84/Ca ratios (yellow) with SST (pink) between June 1992 and August 2004. Gaps in the lines for Mg and Sr correspond to the fractures in the shell (see Figure 20). SST Data sets were provided by the Terrestrial-Environmental Sciences Program of the Smithsonian Tropical Research Institute.-STRI (http://striweb.si.edu/esp/physical_monitoring/download_orts.htm).

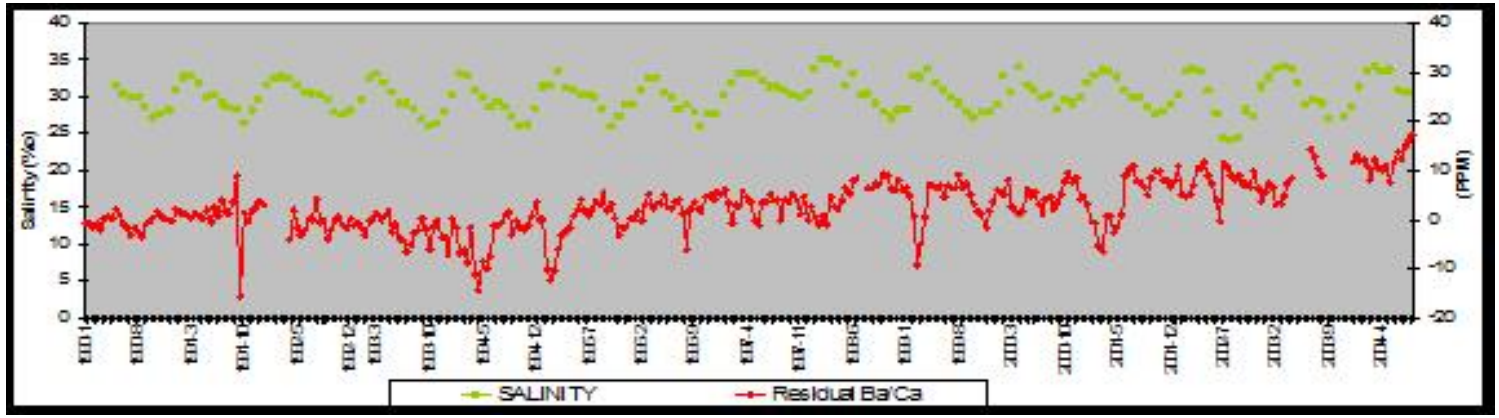

Figure 41. Potential relation between residuals of Ba/Ca ratios and salinity between May 1990 and August 2004. Gaps in the line for Ba correspond to the fractures in the shell (see Figure 20). Salinity data was provided by the Smithsonian Tropical Research Institute.-STRI (www.stri.org).

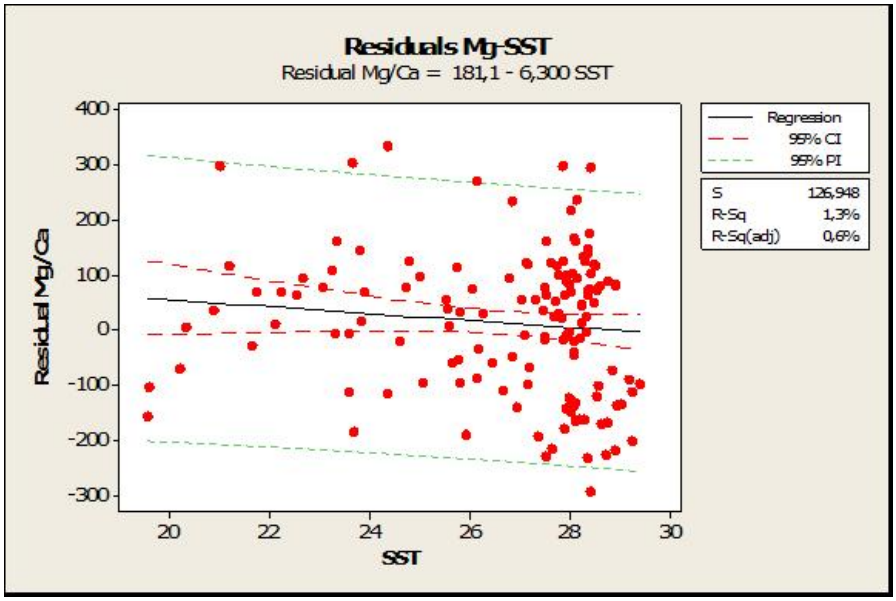

Figure 42. Linear regression analysis between residuals of Mg/Ca ratios and monthly values of SST in Las Perlas Archipelago. SST Data sets were provided by the Terrestrial-Environmental Sciences Program of the Smithsonian Tropical Research Institute.-STRI (http://striweb.si.edu/esp/physical_monitoring/download_orts.htm).

Table 18. Analysis of variance for the linear regression analysis between residuals of Mg/Ca ratios and SST from Las Perlas Archipelago.

Analysis of variance of regression					
SOURCE	DF	SS	MS	F	P
REGRESSION	1	30370	30369.6	1.88	0.172
ERROR	142	2288451	16115.9		
TOTAL	143	2318821			

In the case of Sr, the regression analysis proved that the residuals of Sr/Ca increased with reductions in SST (Figure 43) and, therefore, the assumptions of negative relation between the variables were confirmed. The analysis of variance of the regression (Table 19) showed the highest variance ratio (F=2.66) for Sr from all the techniques (see Tables 18 and 20), and the lowest value of probability to accept the null hypothesis of no relation (P=0.105). The above means that the linear regression was statistically significant to confirm the negative relation between Sr/Ca and SST, although there were data outside of the 95% confidence bands and 95% prediction intervals.

On the other hand, the results of the regression analysis for Ba showed no relation between Ba and the monthly values of salinity (Figure 44). The results were evident in the graphic because most of the data was outside of the 95% confidence bands and some values were outside of the 95% prediction intervals. The “no relation” obtained in this case maybe be due to the trend that remained after the transformation (see Figure 41).

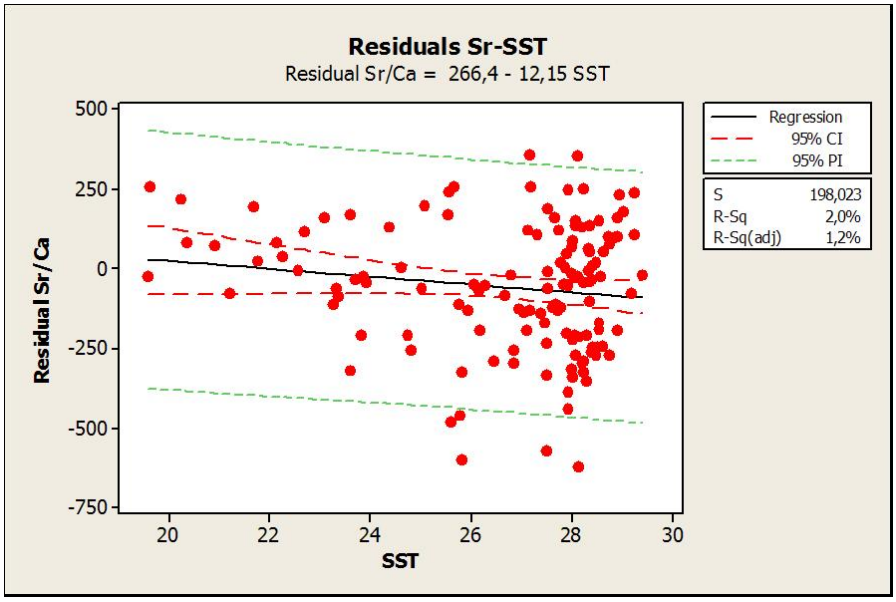

Figure 43. Linear regression analysis between residuals of Sr/Ca ratios and monthly values of SST in Las Perlas Archipelago. SST Data sets were provided by the Terrestrial-Environmental Sciences Program of the Smithsonian Tropical Research Institute.-STRI (http://striweb.si.edu/esp/physical_monitoring/download_orts.htm).

Table 19. Analysis of variance for the linear regression analysis between residuals of Sr/Ca ratios and SST from Las Perlas Archipelago.

Analysis of variance of regression					
SOURCE	DF	SS	MS	F	P
REGRESSION	1	104321	104321	2.66	0.105
ERROR	133	5215431	39213		
TOTAL	134	5319661			

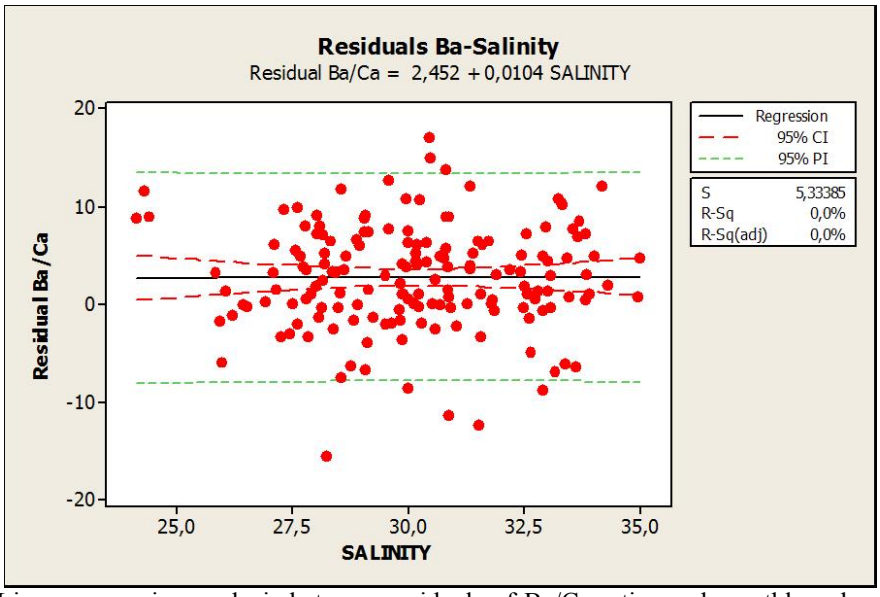

Figure 44. Linear regression analysis between residuals of Ba/Ca ratios and monthly values of salinity from Panama Bay. The analysis of variance of the regression showed null variance ratio (F=0) and a probability higher than 0.5 (P=0.954). The above means that there was not statistical significance for the positive relation described by the equation. Salinity data was provided by the Smithsonian Tropical Research Institute.-STRI (**www.stri.org**).

4.5.10 Oxygen isotopes with Ion Microprobe-IMF:

A total of 47 determinations were collected directly from a transverse section of the selected shell (PAN-04-55-8) and analyzed with the IMF, all of them between the fractures (Fr) observed in the internal layers (see Figure 20 bottom). From the total, 20 values corresponded to line 1, 21 values were in line 2 as complementary to line 1, 18 values were distributed in line 3 to prove reproducibility of lines 1 and 2, and 2 independent values were at the end of line 3 to prove isotopic behaviour of the very strong annual bands.

Results of the analysis (Figure 45) showed values between -4.69 and -2.20 PDB ($\Delta\delta^{18}O$ aragonite -2.49PDB), with an average of -3.41PDB. Samples of fossil vermetids from the Mediterranean Sea showed values between 1.18 and 1.94PDB (Silenzi *et al.* 2004). The positive values and the differences relative to those obtained during this work correspond to the reductions in the SST during the period in which the fossils created the shells, 1600 to 1850AD, a period known as little Ice Age (Silenzi *et al.* 2004).

From Figure 45 it was also evident that line 3 follows with good accuracy the results of lines 1 and 2 (L1 and L2). In the same way, points 1 and 2 (P1 and P2) presented similar results to the last point in line 3 (L3). Thus, the overlapping between the lines (as well as P1 and P2 with the last point in L3) proved the reproducibility necessary for the analysis. The plot also showed that some of the peaks (orange circles) were correlated with some of the annual bands and the last El Niño year (band G: 1998) was correlated with low values between oranges circles for bands F and H. The lower values during El Niño year (warmer year) suggested a negative relation between oxygen isotopes and SST, as was exposed previously with the results for oxygen isotopes with MS (see 4.5.4 preliminary oxygen isotopes) and reported by Silenzi *et al.* (2004) for fossil and alive vermetids from the Mediterranean Sea.

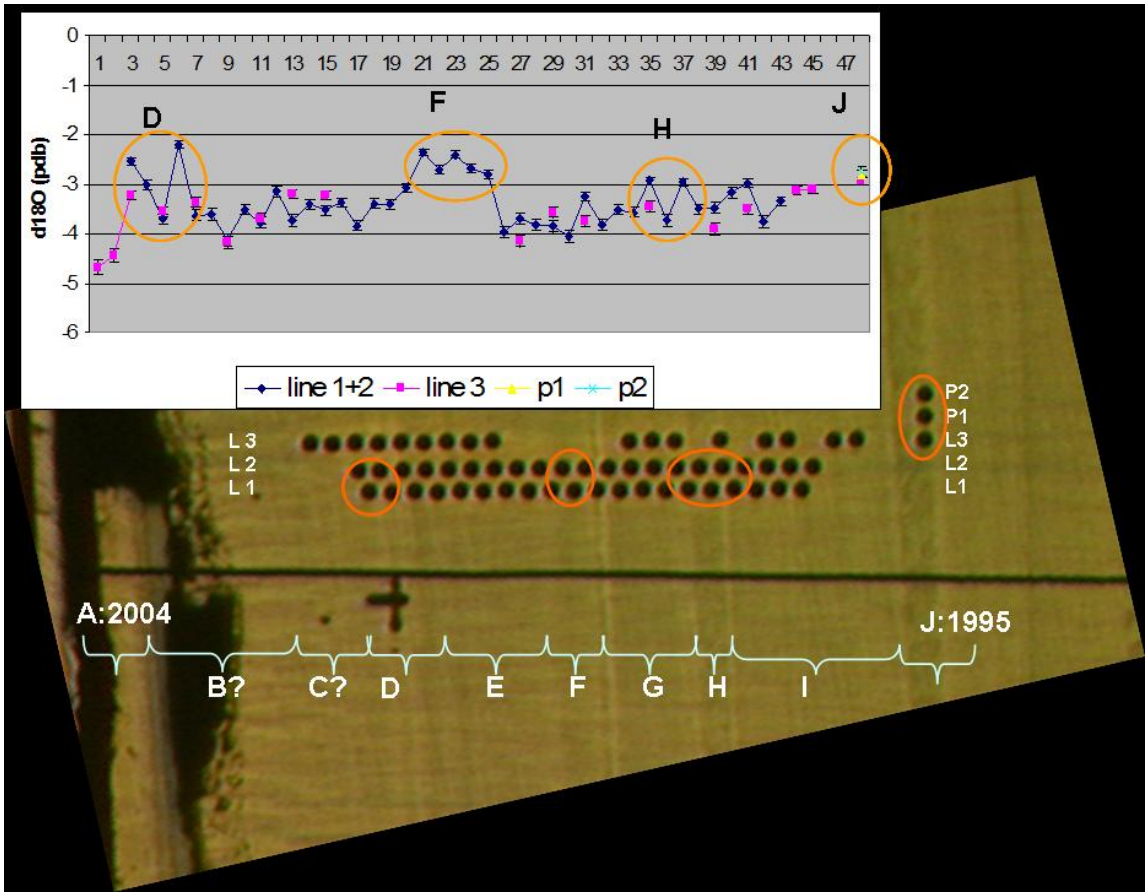

Figure 45. Results of analysis for oxygen isotopes with IMF for samples collected from a transverse section of the selected shell (PAN-04-55-8). L1: line 1. L2: line 2. L3: line 3. P1: point 1. P2: point 2. X axis= number of the samples in a hypothetic line that combined L1, L2, L3, P1, and P2. Y axis= concentration of $\delta^{18}O$ in PDB. The results showed relations between the analysis and the growth bands.

The next step in the analyses was to confirm the isotopic equilibrium between the oxygen isotopes in the shells and the values for oxygen isotopes obtained from water samples collected in the area (see 4.5.5 results of oxygen isotopes in sea water, page 119). Due to the fact that the results of the X-ray diffraction, SEM and EBSC showed aragonite as the main composition of the shells, a modification of the Epstein *et al.* (1953) formula presented by Grossman and Ku (1986) was used. From this formula were obtained the theoretical values of $\delta^{18}O$ in aragonite (for the shell) as follow:

$Y = a - bX$

$SST\ (°C) = 21.8 - 4.69\ (\delta^{18}O_{aragonite} - \delta^{18}O_{water\ in\ VSMOW})$

$SST - 21.8 = -4.69\ (\delta^{18}O_{aragonite} - \delta^{18}O_{water\ in\ VSMOW})$

$$\frac{(SST - 21.8)}{4.69} = -\delta^{18}O_{aragonite} + \delta^{18}O_{water\ in\ VSMOW}$$

$$\frac{(SST - 21.8)}{4.69} - \delta^{18}O_{water\ in\ VSMOW} = -\delta^{18}O_{aragonite}\ (-1)$$

$\frac{(SST - 21.8)}{-4.69} + \delta^{18}O_{water\ in\ VSMOW} = \delta^{18}O_{aragonite}$

$\delta^{18}O_{aragonite} = \frac{SST-21.8}{-4.69} + \delta^{18}O_{water\ in\ VSMOW}$

And including the additional correction to changes values in water from VSMOW to VPDB (see page 62 in Grossman and Ku 1986: $\delta^{18}O_w$ in VPDB= $\delta^{18}O_w$ in VSMOW-0.27)

FORMULA No. 3:

$\delta^{18}O_{aragonite} = \frac{SST-21.8}{-4.69} + \delta^{18}O_{water} - 0.27$

The mean $\delta^{18}O$ in water from the four samples collected in Las Perlas was -0.32 VSMOW (see section b.6 in methodology and results)

The new formula (formula No.3) was then used to obtain the: a) mean, b) maximum and, c) minimum theoretical values in order to know the d) theoretical range of variation of the values obtained with the analyses (theoretical $\Delta\delta^{18}O_{aragonite}$= $\delta^{18}O_{aragonite}$ max.- $\delta^{18}O_{aragonite}$ min.) by replacing SST with the average, maximum, and minimum SST in Las Perlas Archipelago between June 1992 and February 2005 from records collected at STRI.

a) Average SST in Las Perlas Archipelago between June 1992 and February 2005 from records collected at STRI (26.63°C):

Average theoretical $\delta^{18}O_{aragonite} = \frac{26.63-21.8}{-4,69} + (-0.32) - 0.27$

Average theoretical $\delta^{18}O_{aragonite}$= -1.02985 – 0.59 = **-1.620** VPDB

Average theoretical $\delta^{18}O_{aragonite}$= -1.02985 – 0.32 = **-1.35 ‰**

b) Maximum SST in Las Perlas Archipelago between June 1992 and February 2005 from records collected at STRI (29.39°C average daily values for June 1998):

Maximum theoretical $\delta^{18}O_{aragonite} = \frac{29.39-21.8}{-4.69} + (-0.32) - 0.27$

Maximum theoretical $\delta^{18}O_{aragonite}$= -1.6183 – 0.59 = **-2.2083 VPDB**

Maximum theoretical $\delta^{18}O_{aragonite}$= -1.6183 – 0.32 = **-1.94 ‰**

c) Minimum SST in Las Perlas Archipelago between June 1992 and February 2005 from records collected at STRI (19.58°C average daily values for March 1997):

Minimum theoretical δ^{18}Oaragonite= $\frac{19.58-21.8}{-4.69}$ + (-0.32) -0.27

Minimum theoretical δ^{18}Oaragonite= 0.4733 -0.59= **-0.1166 VPDB**

Minimum theoretical δ^{18}Oaragonite= 0.4733 – 0.32 = **0.15 ‰**

d) Theoretical $\Delta\delta^{18}$Oaragonite= δ^{18}Oaragonite max.- δ^{18}Oaragonite min.

Theoretical $\Delta\delta^{18}$Oaragonite= -2.21- (-0.12)

Theoretical $\Delta\delta^{18}$Oaragonite= -2.21 + 0.12= -2.09

Theoretical $\Delta\delta^{18}$Oaragonite= **-2.1 (with values for correction to VPDB)**

Theoretical $\Delta\delta^{18}$Oaragonite= -1.94 - 0.15= -2.09

Theoretical $\Delta\delta^{18}$Oaragonite= **-2.1 (without the correction to VPDB, only ‰)**

The above means that the theoretical $\Delta\delta^{18}$O aragonite (shell samples) in the measurements with IMF should be around -2.09PDB. However, the values obtained with the IMF analysis ($\Delta\delta^{18}$O aragonite -2.49PDB) were higher (-0.40PDB, Table 20) than the theoretical values. The difference between theoretical and real values for oxygen isotopes has been established by the scientific and technical staff at Edinburgh University (Ion Microprobe Facility) and they are currently working on a formula to correct the difference (Dr. John Craven Com. Pers.). Results of Silenzi *et al.* (2004) for fossil vermetids from the Mediterranean Sea showed isotopic equilibrium between the isotopic composition of the shells and the sea water. However, since they used the original formula of Epstein *et al.* (1953), without the correction of Grossman and Ku (1986) for samples composed of aragonite, their results may include some analytical error.

Comparisons between the average values obtained with IMF analysis and average theoretical value showed a difference of -1.79PDB (Table 20). Comparisons between the average theoretical value and the average values obtained from MS (-2.23PDB, see 4.5.4 preliminary oxygen isotopes, page 115) showed a difference of -1.18PDB (smaller difference but MS included only 1 year of analysis). The meaning of these results was that, at least under the applied techniques, the samples collected from the shell seemed not to be in isotopic equilibrium with the sea water. However, the vermetid shells were nearer to the isotopic equilibrium than samples from coral skeletons that present values for oxygen isotopes between -5 and -3 PDB (e.g. Tudhope *et al.* 1997) and similar in

isotopic composition to samples of bivalves (see M. Elliot projects at www.geos.ed.ac.uk/homes/melliot2).

Table 20. Comparisons between values obtained with IMF and theoretical values.

	Theoretical values	Results from analysis	Difference
Maximum	-2.21VPDB (-1.94‰)	-4.69PDB	-2.48PDB
Minimum	-0.12VPDB (0.15‰)	-2.20PDB	-2.08PDB
$\Delta\delta^{18}O$ aragonite	-2.09VPDB (-2.24‰)	-2.49PDB	-0.40PDB
Average	-1.62VPDB (-1.35‰)	-3.41PDB	-1.79PDB

To identify historical statistical differences in the values (potential El Niño, La Niña years) other values per year were obtained by using the formula "Y= a + bX+ c" to obtain the slope of the graphic (slope=1/b) as follow:

SST (°C) = 21.8 - 4.69 ($\delta^{18}O$aragonite- $\delta^{18}O$water)

Where b= -4.69

Slope then= 1/b= 1/-4.69

Slope= -0.21‰

The above means that any increase in 1°C should be reflected in a reduction of oxygen isotopes in the shell in 0.21‰. From this proportion the theoretical ΔSST was obtained as follow:

1°C ≈ -0.21‰

Theoretical Δ SST ≈ Theoretical $\Delta\delta^{18}O$ aragonite in ‰ (-2.24, see Table 25)

$$\text{Theoretical } \Delta \text{ SST} = \frac{-2.24‰ * 1°C}{-0.21‰}$$

Theoretical ΔSST= 10.67 °C

The real Δ SST (°C) was obtained as follow:

Real Δ SST (°C) = max. monthly SST (records STRI)-min. monthly SST (records STRI)

Real Δ SST (1992 to 2005)= 29.39°C -19.58°C

Real Δ SST (1992 to 2005) = 9.81°C

Therefore, the theoretical and real Δ SST were very similar and the only deviation from the potential relation between the oxygen isotopes values and SST would correspond to the differences between the theoretical and real values in oxygen isotopes.

In order to obtain new oxygen isotope values, and based in the relation 1°C ≈ -0.21‰ exposed previously, the differences between monthly SST values were used as follow:

Est. value (PDB)= calculate value of previous month(PDB)-((Δ SST of actual/previous month*0.21)+correction from ‰ to PDB)

$\delta^{18}O$ for 10-2002 (PDB)= -2.8190 – ((4.0291*0.21)+0.27)

$\delta^{18}O$ for 10-2002 (PDB)= **-3.9351 PDB**

Comparisons between the isotopic concentrations (including the extra values obtained from the relation 1°C ≈ -0.21‰ exposed previously) and SST for the period 1995-2002 (see Figure 45, period of oxygen isotope analysis) showed that in general there was a negative relation, except during 2000 because the signal did not show the reductions in SST at the beginning and end of the year (Figure 46). There were also negative relations between SST and Sr with the same technique (IMF) and the same chronological period (see Figures 40, 43, 46) and, therefore, a direct relation between $\delta^{18}O$ and Sr could be suggested.

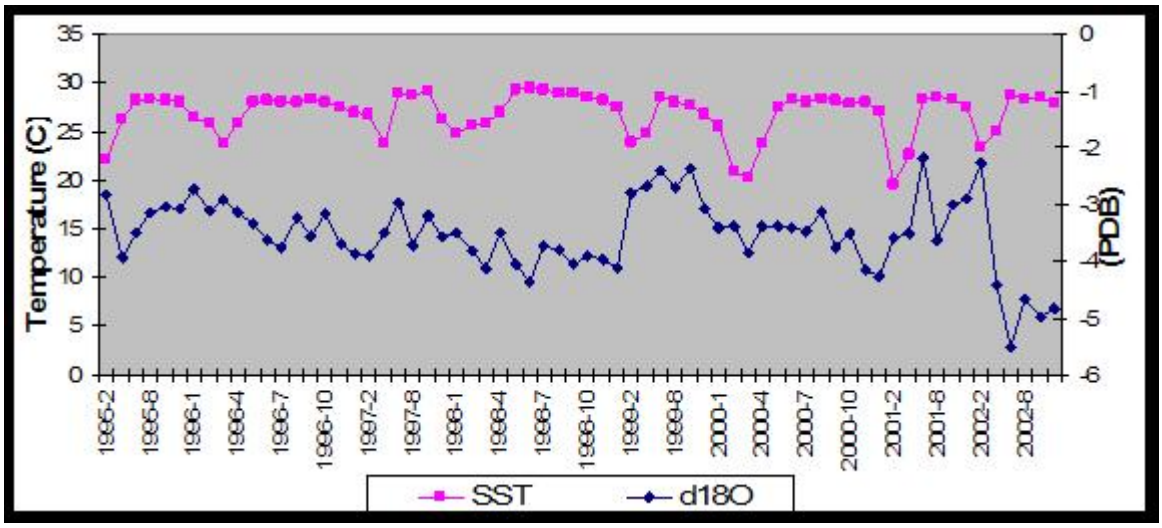

Figure 46. Potential relation between SST and values of oxygen isotopes for the period February 1995 – December 2002. SST Data sets were provided by the Terrestrial-Environmental Sciences Program (**http://striweb.si.edu/esp/physical_monitoring/download_orts.htm**) of the Smithsonian Tropical Research Institute.-STRI

Results of the regression analysis proved that $\delta^{18}O$ increased with reductions in SST (Figure 47) and, therefore, the assumptions of negative relation between the variables were confirmed. The analysis of variance of the regression (Table 21) showed a probability much lower than 0.5 (P=0.148) that confirmed the alternative hypothesis of relation between the variables. The above means that the linear regression was statistically significant to confirm the negative relation between $\delta^{18}O$ and SST, although there was data outside of the 95% confidence bands and 95% prediction intervals.

Table 21. Analysis of variance for the linear regression analysis between $\delta^{18}O$ and SST from Las Perlas Archipelago.

Analysis of Variance for regression between SST and $\delta^{18}O$					
SOURCE	DF	SS	MS	F	P
REGRESSION	1	0.8526	0.852582	2.15	0.148
ERROR	64	25.3910	0.396734		
TOTAL	65	26.2435			

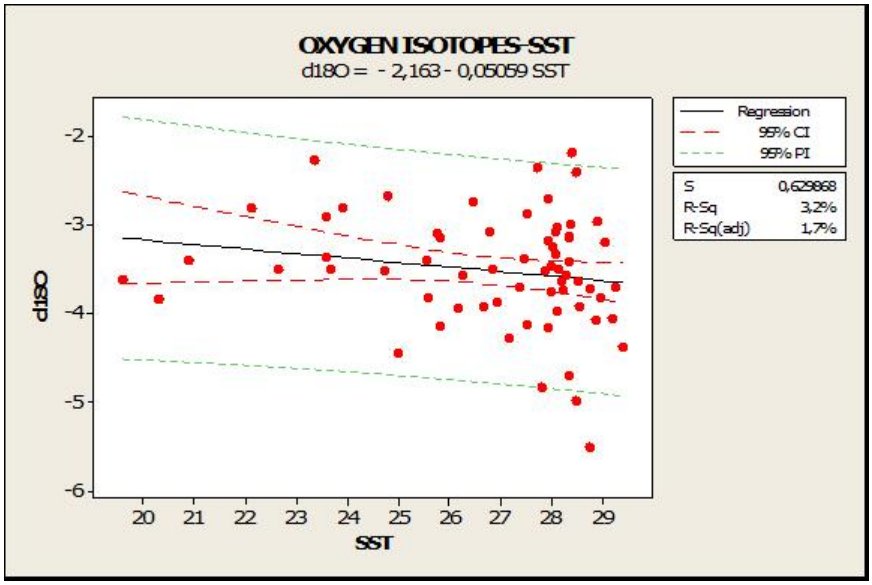

Figure 47. Linear regression analysis between $\delta^{18}O$ and monthly values of SST in Las Perlas Archipelago. SST Data sets were provided by the Terrestrial-Environmental Sciences Program of the Smithsonian Tropical Research Institute.-STRI (http://striweb.si.edu/esp/physical_monitoring/download_orts.htm).

4.6 Comparisons between techniques for trace elements (ICP-OES, LA-ICP-MS, IMF) and between techniques for Oxygen isotopes (MS, IMF):

4.6.1. Trace elements:

Analyses of trace elements (Sr, Mg, and their relationship with SST, Ba and its relationship with salinity) using three different methodologies (ICP-OES, LA-ICP-MS, IMF) were performed in order to identify variations that may be correlated with changes in environmental conditions, and to identify the best methodology for the analysis of trace elements with this proposed proxy indicator. LA-ICP-MS and IMF showed detectable amounts of the three elements, with concentrations of 7-50 ppm (LA-ICP-MS) and 125-740ppm (IMF) for Mg, 63-99ppm (LA-ICP-MS) and 1433-3579ppm

(IMF) for Sr, and 3-17ppm (LA-ICP-MS) and 14-40ppm (IMF) for Ba (see Figures 33 and 39). ICP-OES showed detectable amounts of Mg and Sr with concentrations of 0.3-1.2 ppm and 1.7-2.4 ppm respectively (see Figure 28); Ba was under the limits of detection of the machine. Thus, there were differences in the obtained values between methodologies and even some times within a same methodology for a single element (see Figure 32). The only consistency was their proportions: Sr concentrations > Mg> Ba concentrations. Therefore, the only way to compare between methodologies was to see the general patterns. Differences in the concentrations of the elements and in even in proportions between elements have been found by other authors for a single group of corals (*Porites*). Mitsuguchi *et al*. (1997) found that the concentrations of Mg/Ca ratios were 4 times higher than the concentrations of Sr/Ca ratios. On the other hand, Fallon *et al.* (2003) found concentrations of Sr/Ca ratios twice as higher as the Mg/Ca ratios.

Based on the general patterns obtained with the three methodologies:

- Mg/Ca ratios seem to have a positive relationship with SST (see Figures 29 and 36), although in one case it was not statistically significant (see Figure 29).
- Sr/Ca ratios seem to have a negative relation with SST (see Figures 30, 37, 43), although in one case it was not statistically significant (see Figure 30).
- Ba/Ca ratios seem to have a negative relation with salinity (see Figure 38: not statistically significant; Figure 43: positive relation but not statistically significant and with a permanent trend in the data), although the results were not statistically significant.

The relationships obtained between Sr and Mg with SST and Ba with salinity show agreement with relations found between the elements and the two variables for coral species (e.g. McCulloch *et al.* 2003, Fallon *et al.* 2003). Obviously, there are exceptions (e.g. Villiers *et al.* 1994).

When one compares the three methodologies (Table 22) with respect to the temporal and spatial resolution required for the analysis in the species (annual bands: 121µm, thickness of the shell: 2mm), the time for preparation of samples for the analysis (exponential increasing time for preparation under solution in case of ICP-OES), and the facility to access a good volume of sample (which is much bigger in the case of ICP-OES, or even LA-ICP-MS), ICP-OES showed to be impractical in terms of sampling and IMF seemed to be more expensive in the short time (Table 22). On the other hand,

ICP-OES seemed to have more accuracy in the results than IMF and LA-ICP-MS. Therefore, in terms of accuracy, ICP-OES analysis was shown to be the best methodology to analyze annual growth bands in the vermetid with a higher accuracy and lower cost. In order to access the minor bands (more detail), to collect minimal amount of samples for the analyses, and to be able to compare the results of the analysis with the band pattern from where they were collected (pictures of samples analyzed), Ion Microprobe Analysis-IMF was the most practical methodology.

Table 22. Comparison of different methodologies for sampling the vermetid *E. Tulipus*. *spatial resolution in IMF refers to the diameter of beam, in LA-ICP-MS diameter of laser/depth, and in ICP-OES/MS to the minimal depth/diameter of drilling to collect samples (in these cases it was essential to access sample in different way). Amount of sample to collect and correction of human error are not applicable (NA) in IMF and LA-ICP-MS because the sample were taken directly by the machine and related to the spatial resolution defined a priori, that is the reason why it was essential to have in ICP-OES corrections for both, human and equipment errors. ¥cost of one day of IMF (more than 40 analysis) plus £20 for preparation of samples (one polished block). **Calculated with a minimal of 40 samples and without the drilling process. The time will increase with the number of samples.

FEATURE	IMF	LA-ICP-MS	MS	ICP-OES
Detectable elements	Mg, Sr, Ba, $\delta^{18}O$	Mg, Sr, Ba	$\delta^{18}O$	Mg,Sr
Max. spatial resolution (µm)*	5 (trace elements) 40 ($\delta^{18}O$)	75x20	250x500	250x500
Accuracy	1% (trace elements) 0.2-0.5% ($\delta^{18}O$)	6-9%	1%	0.7%
Min. amount sample	NA	NA	0.1-0.3mg	3-5 mg
Correction equip. error	standards	standards	Standards/ blanks	Standards/ blanks
Correction human error	NA	NA	Through weight of standards	Through weight of standards
Cost per analysis	£420/day¥	£25/sample	£20/sample	£20/sample
Prep. Samples (hours)	8	8	2-4	Min 20**

4.6.2. Oxygen isotope analysis:

Analysis of oxygen isotopes in the shell ($\delta^{18}O$/ $\delta^{16}O$ ratios in PDB) under two different methodologies (IMF, MS) were performed in order to identify variation that may be correlated with changes in environmental conditions. MS showed detectable accumulation with concentrations between -2.7 and -1.3 PDB (Figure 22). IMF also showed detectable accumulations with concentrations between -4.7 and -2.2 PDB (Figure 45). Here again there were differences in the values between methodologies and within each methodology for analysis in the same band or chronological range (see Figures 22 and 45) and, therefore, the only way to compare between methodologies was to see the general patterns (shape of the graphics). In this case, and contrary to the trace elements analysis, isotopic composition in corals has proven to be very consistent, with similar ranges (-5 to -3 PDB) even between different coral species (e.g. Grottoli 1999, Watanabe *et al.* 2001, Linsey *et al.* 2004). Comparisons in the patterns obtained for corals and this vermetid species showed that in both cases, Corals and *E. Tulipus*, there is a negative relation between $\delta^{18}O$ and SST (e.g. e.g. Grottoli 1999, Watanabe *et al.* 2001). Comparisons between the values of $\delta^{18}O$ from this work and those obtained by Silenzi *et al.* (2004) were difficult because the latter corresponded to fossil records during the Little Ice Age, a period with general reductions in SST. Besides, results with the IMF for oxygen isotopes and trace elements suggested a positive relation between $\delta^{18}O$ and Sr (see Figures 40, 43, 46)

When IMF and MS were compared with respect to the temporal and spatial resolution required for the analysis in the species (annual bands: 121µm, thickness of the shell: 2mm), the time for preparation of samples for the analysis (exponential increasing time for preparation of samples in MS), and the facility to access a good volume of sample from the desirable band (better in IMF), MS seemed to be impractical in terms of time for preparation and facility to access a measurable volume of sample, but showed a good accuracy and lower cost than IMF, and the precision can increase when used for general analysis (see Table 22). Therefore, MS is recommended for the analysis of general growth band patterns. As in the case of trace elements, IMF was very useful to obtain more detail and it was the only tool in which it was possible to compare the results of the analysis with the band pattern from where they were collected.

In terms of analytical requirements and feasibility to perform IMF, the results showed that although there is less accuracy in the oxygen isotope than in trace elements analyses, due to the beam size of the ion-microprobe (approximately 40 and 20 μm, respectively), there are evident concentrations of all the elements. Indeed, there was a high reproducibility between analysis of the same element in a same sample or block and it was possible to suggest correlations between the results for oxygen isotopes and trace elements (see Figures 40, 43, 46).

All previous results and comparisons showed that: 1) vermetids seem to be a good potential proxy indicator of SST and potentially of salinity, although the last one must be confirmed with further analysis. 2) it is reasonable to assume that more analysis of isotopes and trace elements in this vermetid from other areas would help to confirm it as a potential indicator for the wider TEP region.

4.7. Conclusions:

The shells from Las Perlas Archipelago (Panama) showed an annual longitudinal growth rate of 24mm and detectable annual, monthly and daily bands.

X-ray diffraction and SEM analysis showed aragonite as the main shell component.

Concentrations of oxygen isotopes in water showed similar results to those reported previously for the area. Comparisons between shells and water oxygen isotopes showed that accumulation of oxygen isotopes in shells are not in isotopic equilibrium with the sea water but are nearer to the isotopic equilibrium than corals.

Analysis of trace elements (Sr, Mg, and Ba) and oxygen isotopes using different methodologies showed that there was detectable accumulation of the elements. Analysis of trace elements showed a positive relationship between the Mg signal and SST and a negative relation between Sr signal and SST. There seems to be a negative relation between the Ba signal and salinity but it is necessary to perform more analysis in order to confirm its significance.

The relationship between the trace elements and the records for SST and salinity are in general agreement with previous literature for other marine invertebrates. The results

also showed a negative relation between $\delta^{18}O$ and SST that is in general agreement with previous literature for other marine invertebrates.

Comparisons between methodologies for trace elements showed ICP-OES as the best methodology to analyse annual growth bands in the vermetid, with a high accuracy and low cost. When more detail and/or minimal amounts of sample are needed IMF is the most practical methodology. Besides, it gives the option to compare the results of the analysis with the band pattern from where they were collected.

Comparisons between methodologies for oxygen isotopes showed that MS was reliable to analyze general growth band patterns, but IMF was better to obtain more detail and been able to compare the results of the analysis with the band pattern from where they were collected.

PART 3. SABELLARIDS

Chapter 5. Distribution of *Idanthyrsus cretus* (Sabellaridae:Polychaeta) in the Tropical Eastern Pacific-TEP and application of PCR-RAPD's tools for population analysis

5.1. Abstract:

Sabellarid polychaetes, commonly known as honeycomb worms, can form large reef-like aggregations in many parts of the world. One species, *Idanthyrsus cretus,* has recently been found in several localities during surveys of the Las Perlas Archipelago, in the Tropical Eastern Pacific-TEP of Panama. Large Sabellarid colonies were only found in the south-western area of the archipelago in areas of high exposure. The distribution patterns were examined and mapped with the aid of GIS. Large reefs of this species have not been recorded elsewhere for the Tropical Eastern Pacific-TEP. Principal Component Analysis-PCA shows that the distribution of polychaete reefs in Las Perlas Archipelago and the reef building itself seem to be mainly dependent on depth, a suitable substrate and topography, the potential particle size for tube building, and wave exposure. Comparisons with associated fauna evidence that *I cretus* reefs in the south part of the archipelago may be offering physical and ecological space to a big diversity of associated fauna that in other parts of the archipelago are provided by the coral reefs. Statistical analysis (software GELCOMPARE, cluster with similarity based on Pearson correlation coefficient and UPGMA dendrogram type) of PCR-RAPD's results shows that the polychaete reefs in Las Perlas Archipelago correspond to one population (group of larvae or invasion of a colony) that migrated from the south, probably Colombia or Ecuador. To confirm the reef origin it would be recommend that complementary PCR-RAPD's analysis is carried out with specimens from those countries.

5.2. Introduction:

The Sabellarid polychaete, *Idanthyrus cretus* Chamberlain, 1919 was first described from Taboguilla Island (Pacific of Panama) and as *Idanthyrus regalis* Chamberlain, 1919 from the Galapagos Islands. The two species were recently synonymised by Kirtley as *I. cretus* (Kirtley, 1994). Since the original description, many *Idanthyrus cretus* specimens have been collected from the Pacific coast of Colombia (Hartman,

1940), Costa Rica (Hartman, 1944), the Galapagos Islands (Hartman, 1944; Kirtley, 1994), Ecuador (Hartman, 1944) and other localities in Panama (Monro, 1933; Fauchald, 1977; Kirtley, 1994). Locality details, conditions for settlement and reef building for *I. cretus* are largely unknown as they were not recorded (Table 27).

There are 17 *Idanthyrus* species from around the world. Many taxa have been misidentified due to lack of appropriate literature and access to collections. Most *I. cretus* specimens had been identified as *Idanthyrus pennatus* Peters, 1854, which was originally described from Madagascar, or *Idanthyrus armatus* Kinberg, 1867. A revision of the Sabellaridae family (Kirtley, 1994) was published and many records of Sabellarids were re-identified and described as new species. The revision provided a synopsis of the 12 genera and 111 species and included a summary of the ecological requirements and a review of distribution patterns for each species.

Table 23. Distribution of *I. cretus* in TEP and main habitat details.

Reference	**Locality**	**Original identification**	**Kirtley's identification**	**Habitat details**
Chamberlain, 1919 p485	Panama, Isla Taboguilla	*I. cretus* The holotype.	*I. cretus*	Albatross St.? 1.8m coral.
Monro, 1933 p1065	Panama, Taboga Island,	*I. pennatus*	*I. cretus*	Hotel pier
Monro 1933 p1065	Panama, Gorgona Island,	*I. pennatus*	*I. cretus*	From coral at low tide and at one fathom, in tubes or solitary,
Fauchald, 1977, p54	Panama_Gorgona Island, Taboga lsland, (This is Monro's sample)	*I. pennatus*	*I. cretus*,	all low to mid shore.
Hartman, 1944, p336 RV "Velero"	Panama, Pinas Bay, Secas Island, Coiba Island	*I. pennatus*	*I. cretus*	3.6-7.3m tubes overgrown with coral,
Kirtley, 1994, p95	Panama, Pedro Gonzales Island, Islas Perlas.	*I. pennatus*		
Hartman, 1944, p336,pl31,	Velero exp from Mexico to Ecuador	*I .pennatus* *I. armatus*.	*I. cretus*	Shore to 18fms,

Hartman, 1944 P336 RV "Velero"	Colombia, Puerto Utria, reefs, Octavia Bay	*I. pennatus*	*I. cretus*	outer island, shore, on *Pocillopora* coral. All shallow to 30-35fthms coarse sand and gravel.
Hartman, 1944 p336 RV "Velero"	Costa Rica Playa Blanca, Viradores Islands, Puerto Culebra	*I. pennatus*	I. *cretus*	on *Poronia* coral,
Hartman, 1944 p336	Ecuador Punta Santa Elena, Waldo Schmitt coll	*I. pennatus*	*I. cretus*	tide pool
Hartman, 1944,p336 RV "Velero"	Galapagos, Gardner Bay Hood Is, Flamingo Bay Charles Island, Albemarle Sound, Duncan Island, Darwin Bay Tower Island, Sullivan Bay James Island	*I. pennatus*	*I. cretus*	shore
Kirtley, 1994 p95	Galapagos Floreana, Tagos Cove, Chatham Island, Sullivan Bay James Island	*I* sp?	*I. cretus*	Shore and tide pool
Chamberlain, 1919, p 487	Galapagos	*I. regalis* The holotype	*I. cretus*	Chatham Island

It is well known that Sabellarid distribution is constrained by the availability of stable substrates for the settlement of immature stages and sedentary adult stages. All Sabellarids are filter feeders and need an abundant food supply, high levels of dissolved oxygen and suspended sediments or shell fragments for tube building (Wilson 1929, Volvelle 1965). Therefore, they are usually found in the surf zone in waters of less than 10m, although a few species are known from deep water such as *Gesaia* spp, *Phalacrostemma* sp and *Bathysabellaria* sp (Kirtley, 1994). In the past there has been minimal recorded ecological information relating to depth, temperature, salinity, distribution patterns, reproduction, development stages, micro-habitats, associated fauna, and associated habitat details.

There are very few molecular sequences and publications relating to Polychaeta. One of the recent publications that include phylogenetic analyses is related to the species *Osedax rubiplums*, a whale bone-eating worm (Rouse *et al.* 2004). There is also a list of the genera and species of polychaetes, including Sabellaridae, that are reported in the GeneBank (Soosten *et al.* 1998, Schmidt and Westheide 1999).

The aim of this study is therefore to reveal potential distribution patterns for *Idanthyrus cretus* in the TEP of Panama in relation to physical data and the reasons for that settlement and actual distribution. The study includes a molecular approach to the populations analysis and geographical origins of the Las Perlas colonies that attempt to answer the following questions: 1) were the reefs developed from one original colonization or from several events or settlements? 2) Do the stations correspond to one or several populations? 3) Do the stations have a common origin?

5.3. Study area:

The Las Perlas Archipelago, in the Tropical Eastern Pacific-TEP of Panama (Figure 47), is a very productive area due to the existence of upwelling (D´Croz *et al.* 2003). This upwelling leads to shallow thermoclines and, therefore, the enrichment of the coastal and the near-shore waters. However, a remarkable characteristic of the TEP is that concentrations of surface nutrients remain continuously high and never appear to be completely depleted despite the high phytoplankton productivity (Agujetas-Medrano 2004). There is also high turbidity that results in a low biodiversity for some taxonomic groups like corals (Glynn and Mate 1997, D'Croz and Robertson 1997, Diaz *et al.* 2000), in contrast with the Tropical Western Pacific (Glynn 2001a). The upwelling occurs each year during the dry season that lasts from January to April (D´Croz *et al.* 2001), with the coldest period in March (minimum 15 ºC, average 20ºC) and the warmest period (maximum 32 ºC, average 28ºC) between May and July (D´Croz and Robertson 1997, http://striweb.si.edu/esp/physical_monitoring/download_orts.htm). According to the last Darwin Initiative project, in Las Perlas (Heriot Watt University and the Smithsonian Tropical Research Institute-STRI in Panama and for which this study is an integral part), Sabellarid specimens were not found in grab grids. Only two specimens were found at station 29 in association with coral reefs in depths between 3-4m. Most of the organisms were found in the south east of Las Perlas Archipelago. This area is dominated by sand and rocky habitats (Figure 48) and is characterised by high

wave exposure that brings high levels of dissolved oxygen and suspended sediments or shell fragments for tube building (Wilson 1929, Volvelle 1965).

The Darwin Initiative is a small grants programme that aims to promote biodiversity conservation and sustainable use of resources around the world. The Initiative is funded and administered by the UK Department for Environment, Food and Rural Affairs-DEFRA. One of the most important objectives of the Darwin Initiative is to assist countries rich in biodiversity but poor in resources with the conservation of biological diversity and implementation of the Biodiversity Convention. In this way the goal of the programme is to involve British expertise in the field of biodiversity, by involving either local institutions or communities in the host country (http://www.darwin.gov.uk/about/).

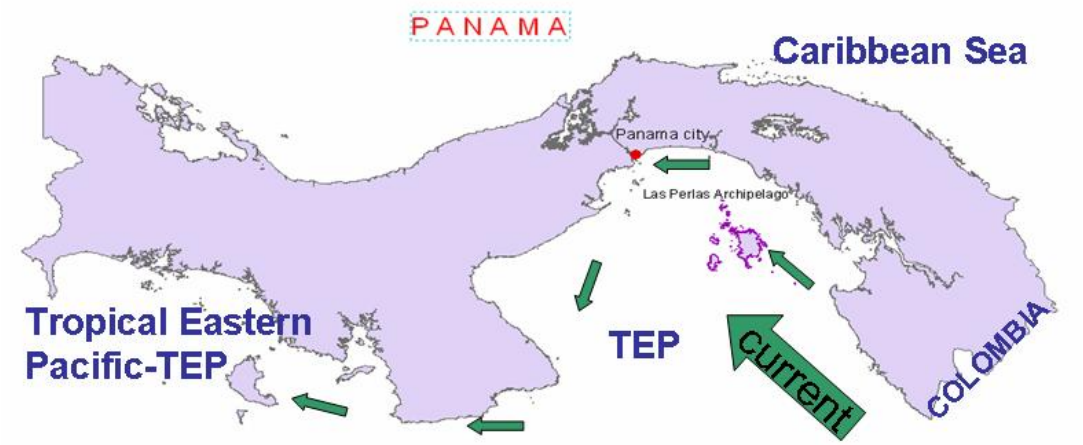

Figure 48. Las Perlas Archipelago in the Tropical Eastern Pacific-TEP of Panama. Arrows show the general current pattern (South Ecuatorial Current-SEC). Modified from Glynn and Mate (1997) and http://www.reef.org/data/database.htm.

5.4. Methodology:

5.4.1. Field work:

The Las Perlas Archipelago is an area to the south east of Panama Bay (8°10'30"-8°42'00"N/ 78°45'45"- 79°06'45"W) and consists of one large, 2-3 medium and several small islands. Primary ecological information (e.g. reef distribution, associated fauna, microhabitat) was obtained from six stations in 2-6m water depth relative to low tide (Figure 48, stations 60, 62, 63, 66, and 68 correspond to a sand habitat type, station 29 corresponds to coral reef habitat type – Darwin Initiative Project Station numbering system). The six stations were selected for detailed study from information collected during several surveys to the area in 2003 and 2004 (Benfield 2005). Station 66, with

the biggest reef, was subdivided into 7 stations, for a total of 12 stations. At each station, at least 10 organisms were removed by cutting off a section of the tube aggregate (blocks of sand tubes; Figure 49), except at station 29 where only two organisms were collected from coral reef environments. The organisms preserved in 10% formalin were identified by traditional methods. Samples preserved in 96% ethanol were analysed with molecular techniques to determine population origin.

Information was also collected relateing to the structure of the polychaete reef (density, dimensions) and the surrounding environments (sediment type, type of benthic community, distribution of the species and percentage cover in coral reef environments by using visual surveys of 30 min). The dimensions were measured with a flexible measuring tape. At the largest reef (St 66 San Telmo) 3 width and 1 length measurements were taken, and 1 width and 1 length in the smaller aggregates. The density was measured in the larger reefs with 1 X 1m quadrants (6 quadrants in total) subdivided in squares of 10X10cm. Additional, secondary information about coral reef environments (e.g. percentage cover of benthic organisms with visual surveys and 1X1m quadrants), and physical variables (e.g. SST records, salinity records) were used for comparison with the information collected during the field surveys.

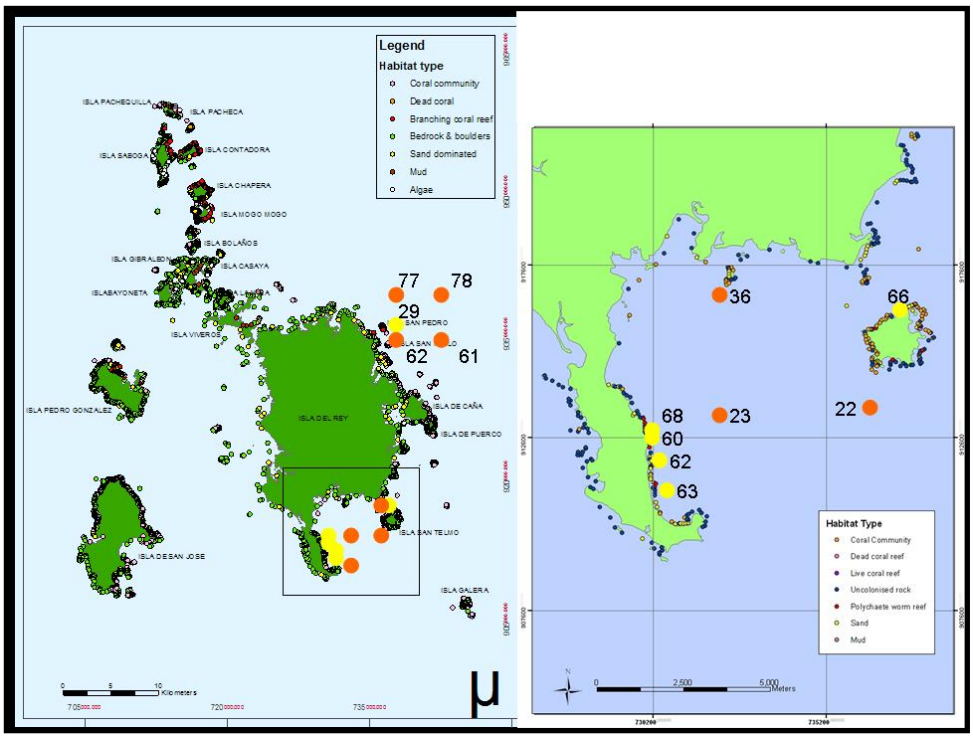

Figure 49. Study area, Las Perlas Archipelago, in the Pacific of Panama and their benthic communities. Circles in Yellow are the 6 stations (60, 62, 63, 66, 68, 29). Circles in orange are the stations for sediments (22, 23, 36, 61, 62, 77, 78 see Annex 4). Courtesy of Sarah Benfield database (Benfield 2005).

Figure 50. Picture of sabellaridae block. Courtesy of the Trustees of the National Museum of Scotland.

5.4.2. Laboratory analysis:

a) Material examined by traditional taxonomy:

The specimens identified as *Idanthyrus pennatus* by Monro, 1933 from Taboga Island (NHM. 1933.7.10.192-196) and Gorgona Island (NHM. 1933.7.10.182-191) were examined.

A total of 50 specimens of *I. cretus* from the stations 60, 62, 63, 66, and 68 (sand habitats types) and 2 organisms collected from station 29 (coral reef habitat type, Figure 18) were described and figured.

b) Molecular analysis:

The molecular analysis was performed at the Centre for Marine Biology and Biotechnology, Heriot-Watt University.

Samples of *Idanthyrsus cretus* collected for molecular analysis (see Figure 48) from a total of 12 stations, including 7 substations in the biggest reef (60, 62, 63, 68, 66.1, 66.3, 66.4, 66.5, 66.6, 66.8, and 66.9 with four replicates, 29 with two replicates, for a total of 46 samples), were prepared for DNA extraction according to the protocol for isolation of total DNA of QIAGEN (QIAGEN 2006) as follows:

1) Cut up to 25mg of tissue into small pieces, place it in a 1.5ml microcentrifuge tube, and add 180µl buffer ATL

2) Add 20µl proteinase k, mix by vortexing, and incubate at 55˚C until the tissue is completely lysed (usually 3h). Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform.

3) Vortex for 15s. Add 200µl buffer AL to the sample, mix thoroughly by vortexing, and incubate at 70˚C for 10 min.

4) Add 200 µl of ethanol (96-100%) to the sample, and mix thoroughly by vortexing.

5) Pipet the mixture from step 4 into the DNeasy mini spin column placed in a 2 ml collection tube. Centrifuge at 8,000rpm for 1 min. Discard flow-through and collection tube.

6) Place the DNeasy mini spin column in a new 2ml collection tube, add 500 µl buffer AW1, and centrifuge for 1 min at 8,000rpm. Discard flow0through and collection tube.

7) Place the DNeasy mini spin column in a 2ml collection tube, add 500 µl buffer AW2, and centrifuge for 3 min at 14,000 rpm to dry the DNeasy membrane. Discard flow-through and collection tube. Remove the DNeasy mini spin column.

8) Place the DNeasy mini spin column in a clean 2 ml microcentrifuge tube, and pipet 200µl buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 8,000rpm to elute. Keep the solution in freezer at -20°C.

PCR-RAPDs protocols, as described by Soosten *et al.* (1998) and Schmidt and Westheide (1999), who worked on a similar project to discriminate worldwide populations of meiofaunal Syllid and Hesionid polychaetes, were used for DNA amplification of the 46 samples (total of 12 stations) with minor modifications. From the original protocols a total of 17 decamer primers OPB (www.mwg-biotech.com, Germany) with a GC-content between 60-70% (Table 28) were used. The reaction volume of 25 µl was composed of 12.5 µl of 2X biomix buffer (Bioline), 0.25 µl of mwg primer, 1 µl DNA, and 11.25 µl DH_2O (MiliQ). Controls were run changing the concentration of DNA for DH_2O. For amplification, one main thermocycler was used (BIORAD, Hercules, California). Forty five cycles were employed, each comprising:
Step 1:94˚C/ 4min (1 cycle), Step 2 (45 cycles): 94˚C/ 1min for denaturation, 34˚C/ 1min for annealing and 72˚C/ 30 sec for elongation (fastest available transition between temperatures), Step 3:72˚C/ 10 min (1 cycle), followed by an endless file at 4˚C/ ∞ (Step 4). (For more information on RAPD-PCR methodology see Soosten *et al.* 1998).

Table 24. Decamer primers (www.mwg-biotech.com, Germany) used and their GC- content.

PRIMER	SEQUENCE 5'-3'	GC-content (%)
OPB-1	GTTTCGCTCC	60
OPB-2	TGATCCCTGG	60
OPB-3	CATCCCCCTG	70
OPB-4	GGACTGGAGT	60
OPB-5	TGCGCCCTTC	70
OPB-6	TGCTCTGCCC	70
OPB-7	GGTGACGCAG	70
OPB-8	GTCCACACGC	70
OPB-10	CTGCTGGGAC	70
OPB-11	GTAGACCCGT	60
OPB-12	CCTTGACGCA	60
OPB-14	TCCGCTCTGG	70
OPB15	GGAGGGTGTT	60
OPB-16	TTTGCCCGGA	60
OPB-17	AGGGAACGAG	60
OPB-18	CCACAGCAGT	60
OPB-19	ACCCCCGAAG	70

Separation of amplified DNA fragments was carried out with the use of gel loading dye (Fermentas Company) at 82 volts for 10min followed by 90 volts at 20-30 min. The ethidium bromide stain was visualized and documented under UV-light. In order to check the best running time for the primer screening, bigger gels were run for a maximum of 2.5 hours (82 volts for 10 min and then 90 volts), taking pictures at 1.5h, 2h and 2.5h. A set of 10,000BP, 1,000BP and 100BP ladders (Fermentas Company) was used for length comparison during the whole procedure. The results were run in a gel of 1% agarose (TAE 1X).

The reproducibility of the data was checked at regular intervals on different levels throughout parallel experiment lines, including potential differences in the amplification patterns that might derive from the use of different thermocyclers (both thermocyclers from the company BIORAD-Hercules-California) as recommended in Soosten *et al.* (1998) (i.e. to use 2 different thermocyclers for a group of samples and their replicates and to use two different gels for a same group of samples that have been processed in the same thermocycler). First, at the beginning of each series the optimal DNA concentration for PCR was determined for the individual animal by testing the PCR with different DNA concentrations per individual (ca. 1, 2 and 4 ng/25 µl reaction volume, completing volume with MiliQ). Pilot experiments by Soosten *et al.* (1998) proved that results were reproducible with up to 25 ng /25 µl reaction volume

(reproducibility with DNA concentrations at 50 ng//25 µl reaction volume was distinctly poor). For the primer screening, and in order to have all the spectrum of possibilities, a combination of all the 46 samples was used (12 stations, 4 replicates per station except in station 29 that had only 2 replicates) with the 3 primers that gave the best results in Soosten *et al.* (1998) and Schmidt and Westheide (1999) analyses (OPB-5, OPB-6, OPB-18). Three samples with the biggest polymorphism expression (larger number of bands) were then selected to compare with the other 14 primers (Table 28). In addition, for each series, one reaction was carried out with a "blind" sample lacking DNA to check the possibility of contamination with foreign DNA.

5.4.3. Statistical analysis:

a) Traditional taxonomy and ecology:

To identify the main environmental factors responsible for the distribution observed for *I. cretus* in the area, Principal Component Analysis-PCA with the software Minitab was used (Wardlaw 2001). The variables included were: depth (metres), percent of sediments (granule, very coarse sand, coarse sand, medium sand, fine sand, very fine sand, silt-clay), wave exposure in categories (1 =sheltered e.g. bay, 2=low to moderate, 3=moderate, 4=moderate to high, 5=high e.g. headland), visibility (metres), percentage of coral cover with respect to total of hard bottoms, topography in categories (this is how complex the bottom – rocky or reef is 1= no vertical structure, very low, 2= low, slight undulations 0-0.5m, 3= Moderate – 0.5-1m outcrops, some boulders, crevices and overhangs, 4 = high, 1.5->2m outcrops, many overhangs, crevices and boulders).

b) Molecular analysis:

The detected amplification product patterns collected from the gels were examined on the GEL-COMPARE software for monomorphic and polymorphic markers (Applied Maths BVBA, 1998; www.applied-maths.com). The GelCompar software determines the degree of polymorphism (number of stable bands in common) by identification of unknown fingerprints, based on statistical occurrence of bands. In this case an identification group of the taxonomic unit is created from the results of the gels, containing a collection of representative fingerprints, representing the variability within the group. The software then counts the relative frequency of occurrence for each individual band. During the identification, more weight is assigned to bands with a high occurrence frequency (typical bands). The program then calculates a horizontal

calibration regression (group of bands in a particular gel vs group of bands in the identification group, which means the other gels saved in the program or a selected group of bands) based on the differences in concentration and distance. In addition, the program calculates a secondary vertical regression based on the differences in morphology and or staining of the successive band groups. Here there is an optimization option that enables the user to align patterns that are not perfectly aligned (e.g. gels of the same sample that ran at a different time but have the same number and type of bands). For the comparative quantification and polymorphism analysis the software has the options of comparative binary tables (0/1 matrix, where 0 absence, 1 presence of a specific band or DNA marker) or tables with absolute band volumes, percentage band areas, distances or real band concentrations. To strengthen the polymorphism analysis, different patterns from the same organisms, obtained by using different primers, restriction enzymes or others, can be combined in one single analysis with a maximum capacity of one million bands that can be compared in a single analysis (1000 bands per single entry, on a total maximum of 1000 entries). The quantification analysis can be based either on 1-dimensional data (derived from the peak areas on the densiometric curves) or on 2-dimensional information (band intensity, band relative surface, concentration, band positions, etc).

Cluster analysis was carried out by the methods of UPGMA (unweighted pair-group method using arithmetic averages), based on the band information provided by the polymorphism analysis. The software offers different coefficients for the cluster analysis (Dice, Jaccard's, and other less common algorithms such as Ward and Neighbour Joining methods) but it has two main coefficients that are recommended: similarity based on Pearson correlation coefficient for clusters on single gels (experiments) and similarity based on averages from experiments for clusters on a combination of gels (experiments). For the analysis the recommended coefficients were used.

5.5. Results:

5.5.1. Traditional taxonomy and ecology:

As part of the first and second year of the Darwin Project twelve different reefs or aggregates of *Idanthyrus cretus* were found during surveys developed by Heriot Watt-STRI in 2003-2004 (Benfield, 2005) in the south-east part of Las Perlas Archipelago, in

the south east of Isla del Rey and at the north of San Telmo Island. During the 2004-2005 survey it was evident that the SE of Isla del Rey was characterised by un-colonised rock surrounded by sand bottoms, while the north part of San Telmo Island is a coral community surrounded by sand bottoms. All the reefs are exposed to strong currents coming from the South East Pacific (which is high in energy and suspended particulates) and waves. Five of the reefs in San Telmo and Isla del Rey, plus two organisms collected in a coral reef station in San Pedro (station 29) were analysed during this work (Table 29). The stations depth range was 0-6m. The top of the reef at San Telmo was exposed at low spring tides. The San Pedro samples (station 29) were found amongst coral and did not form reefs. The area has been surveyed before but no reefs have previously been recorded on this scale.

A description of the bottom structure (different environments) is found in Figure 48. In the figure it is evident that un-colonised rock (blue dots) and sand (yellow dots) are essential elements for the settlement of this species and are the dominant habitats in the South-East of Isla del Rey. Information about sediments in the area is found in Annex 7.

Table 25. General information of Sabellaridae field stations in Las Perlas Archipelago.

STATION.	NAME	LAT	LONG	AVERAGE DEPTH (m)
29	San Pedro	08°27'36.5"N	78°51'08"W	4.2
60	Isla del Rey	08°14.928'N	78°54.665'W	5.4
62	Isla del Rey	8°14.345'N	78°54.564'W	5.4
63	Isla del Rey	8°14.254'N	78°54.564'W	5.4
66	San Telmo	8°17.092'N	78°50.498'W	3.6
68	Isla del Rey	8°15.161'N	78°54.712'W	5.4

The approximate size of the largest reef of *I. cretus* in Las Perlas Archipelago (station 66) was 56 square metres (3.5m average width X 16 m total length) and had an elongated shape parallel to the coast, but the other four reefs had a rounded shape with sizes between 2-4.5 square metres. Within the Sabellaridae family, the reefs are formed from aggregations of individuals of the same species that in some cases reach up to 60 square km (Caline *et al.* 1988). In Florida there are aggregations of *S.floridensis* which have formed reefs 9.6km long and 1 km wide (Kirtley, 1966). It is also known that some reefs (eg *Sabellaria aveolata*) from UK waters can be formed in a few years and also decline quickly (Wilson 1971).

The average density of organisms (Figure 50) in the main *I. cretus* reef (station 66), measured in 38 squares of 10X10 cm was 72.2 organisms/10 square cm, with maximum

values of 116 and minimum of 49 organisms/10 square cm. Observations from the field show that each individual captures food and fragments of rock and shells continuously with the tentacles that also seem to produce the cylindrical tube. The tube apertures is a flat structure or "hood", which are all facing the same direction (Figure 50), and are thought to be protection against strong water flow. The hoods also allow water to flow around the anterior end of the living animal where the feeding tentacles can extend to form a loose "net". In this protected but active particulate region a mucous ball is also formed to capture food which is then directed to the mouth/stoma and digested. Large particles are grasped by the feeding tentacles, held by the prostomial tentacles and directed to the mouth, and finally tested for selection by the lateral lips. Particles are either selected for ingestion, tube building or rejection (Kirtley 1994).

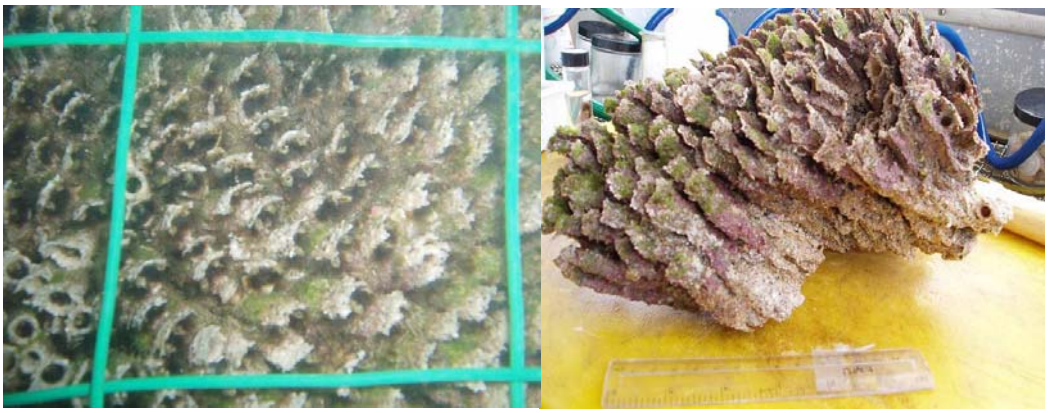

Figure 51. *I. cretus* reef in San Telmo Island (station 66) in Las Perlas Archipelago. a) The square shows and area of 10X10 cm with 64 organisms. b) Block of *I. cretus* reef collected for identification and molecular analysis.

The *I. cretus* reefs also provides a habitat for a diverse fauna of sponges, cnidarians, other polychaeta, molluscs, crustaceans, echinoderms and fishes (Table 30).

Table 26. Number of associated species of each taxonomic group in the *Idanthyrsus cretus* reef in San Telmo Island.

GROUPS/ STATIONS	66.1	66.2	66.3	66.4	66.5	66.6	66.7	66.8	66.9	TOTAL SPP/GROUP
PORIFERA								1		1
CNIDARIA	1	1								2
POLYCHAETA	1			1	5					7
MOLLUSCA	1	5		1				3		10
CRUSTACEA	7	6	1	4	3		1	3	2	27
ECHINO-DERMA	2	5	1	1		1	1		1	12
VERTEBRATA		1								1
TOTAL SPP/STATION	12	18	2	7	8	1	2	7	3	

The body of *Idanthyrus cretus* (Figure 51) is divided into 4 regions, opercular, parathoracic, abdominal and caudal. The main characters are the number of parathoracic segments, shape of the crown (open) and opercular stalk, the type of palae and their distribution (Kirtley 1994).

Figure 52. *Idanthyrsus cretus*, body structure. Courtesy of the Trustees of the National Museums of Scotland.

Morphological descriptions of collected organisms, preserved in 96% alcohol, show that there are two main sizes of polychaetes. The larger polychaetes have a darker colour compared to the smaller ones. The smaller organisms also have ivory coloured bands in each side of the abdominal section. These observations are in agreement with Cazaux (1964), who, in a description for the species *Sabellaria alveolata*, identified sexual differences by external coloration: "the sides of females are a little violet while the males have white sides". Due to the absence of live organisms for these observations we cannot confirm the violet colour in the females but can confirm a darker coloration. Analyses of the internal gonads for each sex showed that:

-Both types of gonads are found in the coelom, around the digestive tube.

-The colour of male gonads were orange and the female whitish. We do not know if the white colour in the female gonads is due to immature eggs.

-Observation of the internal tissue of the male gonads (small organisms) revealed ovoid spermatozoa that are attached to each other by a small branch, giving the appearance of a bunch of grapes. These observations are in accordance with Wilson (1929), who, in a description of the gonads of *Sabellaria alveolata,* mentions that the freshly spawned ova of that species are purplish and with the same shape observed for *I. cretus* male gonads. Our descriptions of male and female gametes are also similar to those of *Sabellaria*

alveolata by Cazaux (1964), who described small spermatozoids with flagella and spheroid female gametes.

Observations of the aggregates show that big tubes with females are surrounded by smaller tubes with males (Figure 52).

Figure 53. Internal structure of *I. cretus* reef. The arrows show the large female tubes surrounded by small male tubes. The small tubes grow in a spiral way around the female tubes.

In the genus *Idanthyrus* there are 17 species from around the world, some of them considered cosmopolitan. Over the last 75 years *I.cretus* has been recorded in the TEP from sites already known for their rocky or coral formations (Kirtley, 1994). The most northerly site is Costa Rica, then moving south along the coastal rim there are several sites in Panama, Colombia and Ecuador (Figure 53, Table 28). The most westerly records are from the Galapagos Islands. These sites are all in the surf zone of the, initially cool Peru Current-PC, that warms as it joins the South Equatorial Current-SEC that is moving toward the equator and to the west (Glynn and Mate 1997). This current is known to carry high levels of sediment. All published records for *I. cretus* (see Table 28) show descriptions of single tubes associated with coral reef environments (e.g. Costa Rica or Colombia) or rocky areas in low to mid shore or tide pools (e.g. Panama, Ecuador, Galapagos). None of these sites have recorded sabellarid reefs as all records are of individual specimens.

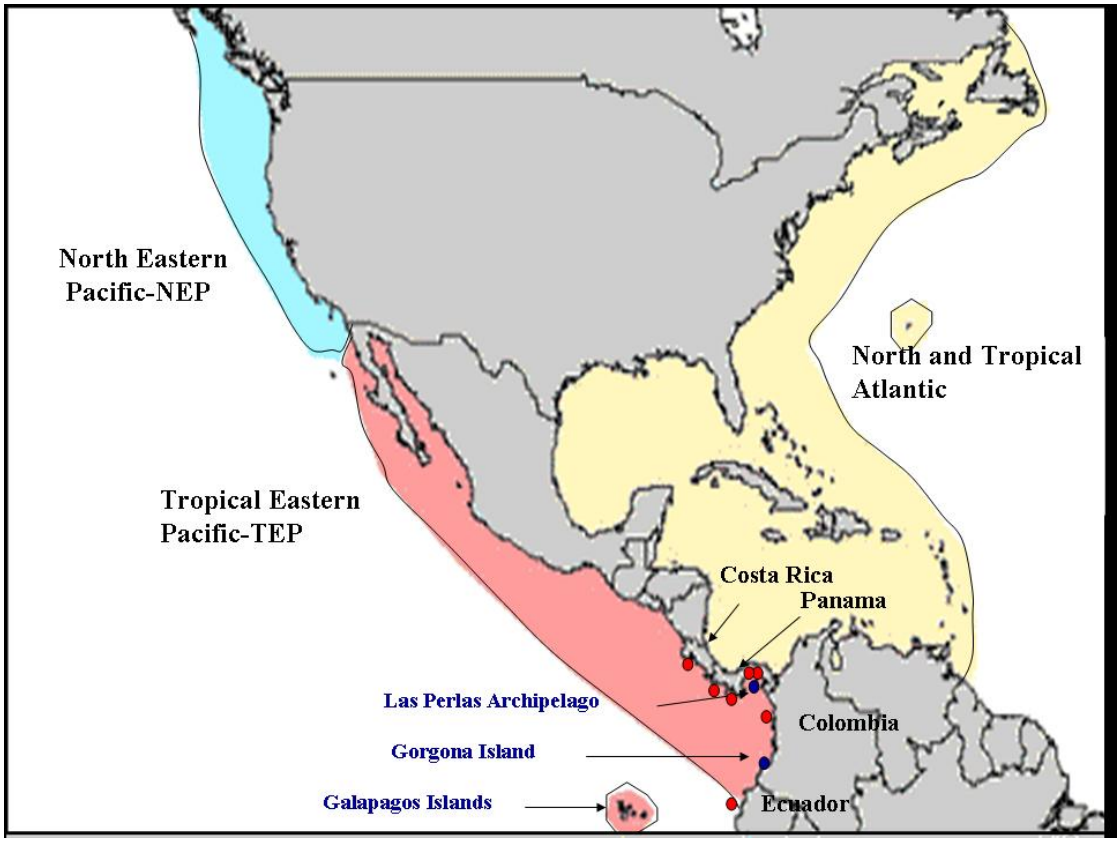

Figure 54. Records of *I. cretus* in the TEP. For more details of records see Table 9. Source http://www.reef.org/data/database.htm.

With respect to the oceanographic conditions of the area, Sea Surface Temperature-SST (Figure 54) and salinity records (Figure 55) collected at Smithsonian Tropical Research Institute-STRI shows that:

1- There is an upwelling between January and April of each year that can be identified by a reduction in temperature (Figure 54) and increase in salinity (Figure 55). The lowest temperatures are in March and the highest between May and July of each year (Figure 54).

2-The historical mean temperature for the last 15 years (1992-2005) in las Perlas Archipelago (see Figure 54) was 26.63°C, but temperatures as low as 14.7°C (Saboga, 22-02-2001) and as high as 30°C (Saboga, 9-11-1997) have been registered.

3- The historical mean salinity for the last 17 years (1990-2005) in Panama Bay (see igure 55) was 30.18‰, but the records show salinities as low as 22‰ (21-10-1995) and as high as 44.8‰ (4-02-2005).

The area also shows continuous high nutrients and phytoplankton (Agujetas-Medrano 2004) creating as a result a high turbidity that causes a low intra-ecosystemic biodiversity for some groups like corals (Diaz *et al.* 2000).

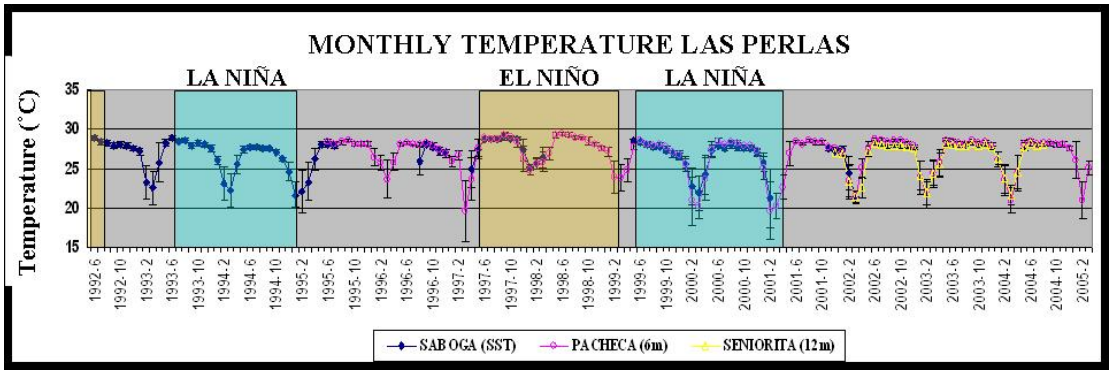

Figure 55. Monthly sea surface temperature-SST for Las Perlas Archipelago during the last 15 years (1992-2005). Data sets were provided by the Terrestrial-Environmental Sciences Program of the Smithsonian Tropical Research Institute.-STRI (http://striweb.si.edu/esp/physical_monitoring/download_orts.htm).

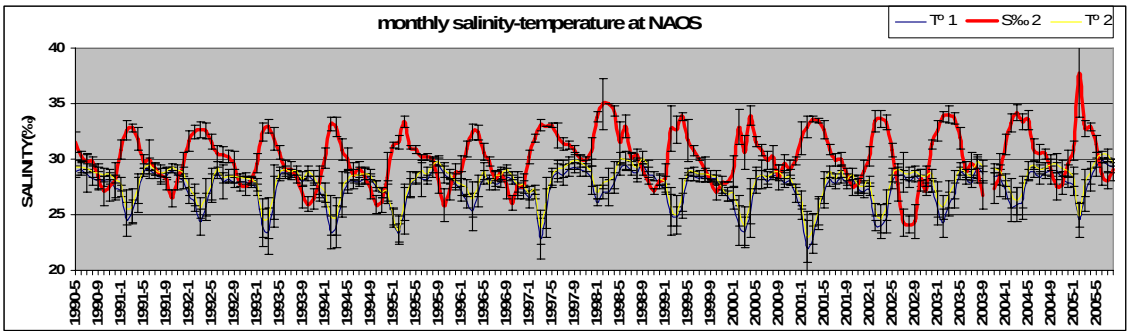

Figure 56. Monthly salinity (in red) and temperature (blue and yellow) during the last 17 years (1990-2005) from Panama Bay. Data sets were provided by the Smithsonian Tropical Research Institute-STRI (www.stri.org).

Results of PCA with the 12 variables (depth, % granule, % very coarse sand, % coarse sand, % medium sand, % fine Sand, % very fine sand, % Silt-Clay, wave exposure, visibility, coral cover, topography) showed two main components that explain in 100% the distribution observed in Las Perlas Archipelago (Figure 56). The first principal component-PC1 has variance (eigen value) of 7.392 and accounts for 61.6% of the total variance. In biological terms, PC1 explains in 61.6% the distribution of the aggregates in the area. The second component-PC2 has variance of 4.608 and explains the other 38.4% of the distribution. The results also show that the PC1 must be a combination of depth, % of granule, wave exposure and topography (see Figure 56, Annex 8) because the coefficients of these variables have the same sign and present the higher values. In the same way, PC2 must be a combination of coral cover and visibility (and in lower grade the proportion of fine and very fine sand in the area). The proportion of the different types of sand (% very coarse sand, % coarse sand, % medium sand, % fine

Sand, % very fine sand) do not seem to be a special factor since they presented values in both components.

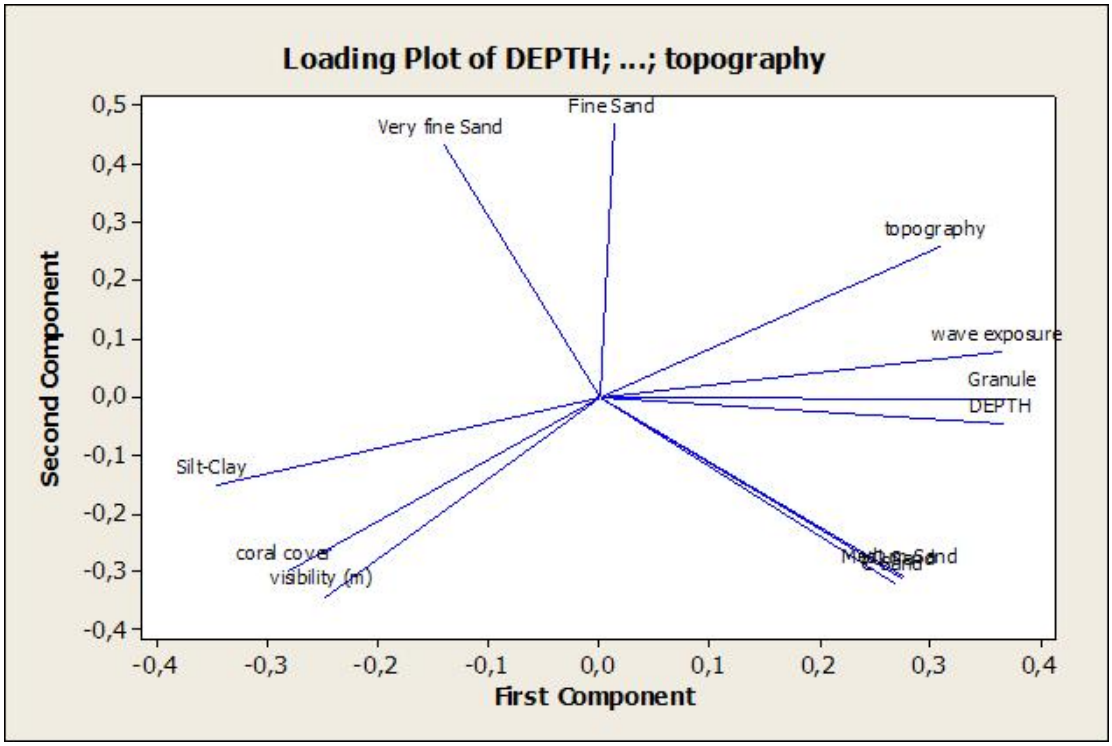

Figure 57. Graphical result of PCA for the first two components that explain 100% of the distribution observed for the polychaetes in Las Perlas Archipelago. Eigen value for PC1 (7.392) explain 61.6% of the distribution of the aggregates. Eigen value for PC2 (4.608) explain the other 38.4% of the distribution (cumulative value = 1). See Annex 5 for complete results of the PCA.

5.5.2. Molecular analysis:

Results of the primer screening showed that:

1) The DNA extraction was successful since there were bands.

2) In order to obtain good gels and pictures for comparisons the optimum period to run small gels (8 pools in one line, 1% agarose) was 25 minutes (82 volts/10 minutes and 92 volts/15 min or until they reached the middle of the gel). In bigger gels (16 pools in one line, 1% agarose, SCIEPLAS Company, www.scieplas.co.uk) 2 hours was a good time (Figure 57). After these times the bands become diffuse and are difficult to identify.

3) The primer screening with OPB-05, OPB-06 and OPB-18 (Figure 57) demonstrated that Primer OPB-18 was better than OPB-6 and both better than OPB-5 because they revealed more bands or polymorphism. Each station presented a different band pattern when compared with the same primer. Station 68 had 5 bands with primer OPB-18, while station 66.1 had 2 bands and station 66.3 between 6 and 7 bands (Figure 57).

However, the last two stations on San Telmo ilustrated slight variation in the bands. Indeed, when comparing the bands with the ladder it was evident that the 4 bands observed in St 66.3 included the 2 bands from St 66.1. These last results were expected due to the fact that stations 66.1 and 66.3 correspond to different parts of the biggest reef in San Telmo Island.

4) The difference between stations 66 (SanTelmo) and 68 (Isla del Rey) seem to prove that those stations correspond to different populations. To confirm differences between stations it was necessary to compare the other stations with all the primers, including the same 3 primers originally selected. Table 31 shows the samples that presented the biggest polymorphism from the primer screen made with primers OPB-5, OPB-6 and OPB18 for the total of samples (46).

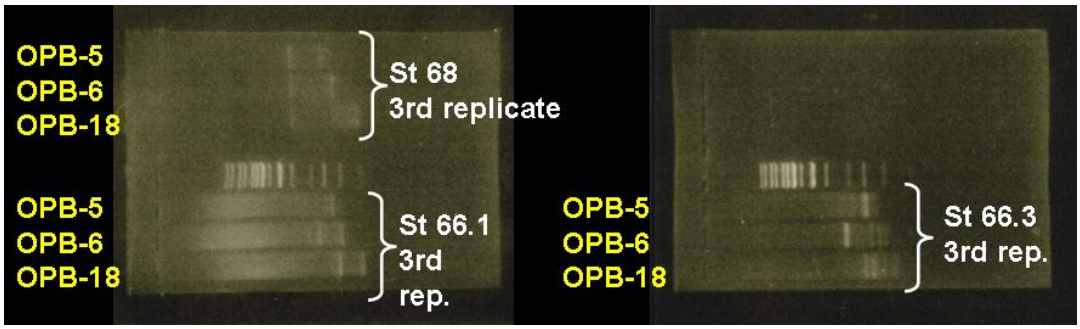

Figure 58. Results of primer screening with decamer primers OPB-5, OPB-6, OPB18. Gel 1 Top left is station 68 from south east Isla del Rey. Bottom left Station 66.1 in San Telmo Island. Gel 2 (right): station 66.3 in San Telmo Island.

Table 27. Samples with the largest number of bands (polymorphism) from the analysis with the first 3 primers: OPB-5, OPB-6, OPB-18. NA: information not available.

Station	replicate	# bands OPB5	#bands OPB6	#bands OPB18
66.3	3	4	6	6-7
62	2	NA	3(+)	7
68	3	3	2(+)	5(+)
66.8	3	1	1	6
66.5	3	1(+)	1	3
66.4	4	NA	NA	4
60	4	NA	NA	4

Samples 66.3.3 (station 66.3, replicate 3) and 62.2 presented the largest number of bands (polymorphism), and therefore were selected to run the primer screen with all the 17 primers (except primer OPB-5 that showed the lowest polymorphism in the first analysis), and to identify the best primers for this species of polychaete. The results of the combination of these two samples with all the primers showed that primers OPB-7,

OPB-8, and OPB-18 gave the best results in terms of number of bands (more bands or polymorphism) and definition or sharpness of the bands (see Figure 57).

In order to run the tests of reproducibility (same RAPD-PCR results running in two different gels and RAPD-PCR results of the same group of samples that have been done in a parallel way in two different thermocyclers and have been run in a same gel) the products of the first RAPD-PCR (Figure 58) were run again in a second gel. In this second case the products of samples 62.2 and 66.3.3 were run again in a second gel with all the primers, except primers OPB-2 (Figure 58, pools 2 and 20), OPB-4 (Figure 58, pools 4 and 22), OPB-19 (Figure 58, pool 17) and OPB-15 (Figure 58, pool 31) that gave poor results in the first gel. The pools were completed with RAPD-PCR products for OPB-18 with samples of stations 68, 66.3.3 and 66.1.3 (See Figure 57) that had been running in a different thermocycler. The results of this new test confirmed OPB-7, OPB-8, and OPB-18 as the best primers to run analysis with this species (Figure 59). The test also confirmed the bands observed the first time (see Figure 57) for samples from stations 68 (Figure 59, pool 29), 66.3.3 (Figure 59, pool 30), and station 66.1.3 (Figure 59, pool 31). In this second case the sample from station 68 (Figure 59, pool 29) had 5 or more bands again, with two stronger bands. Station 66.3.3 (pools 4 and 30, different thermocyclers) had 6 bands. Station 66.1.3 (pool 31) had 4 or more bands with two very strong bands. The last pool (32) corresponds to station 66.9.2 that had been run before and had presented only 1 band. The bands in the pool 1 (control) are the result of a leak or contamination from pool 4 and is the reason why the bands appeared to be similar.

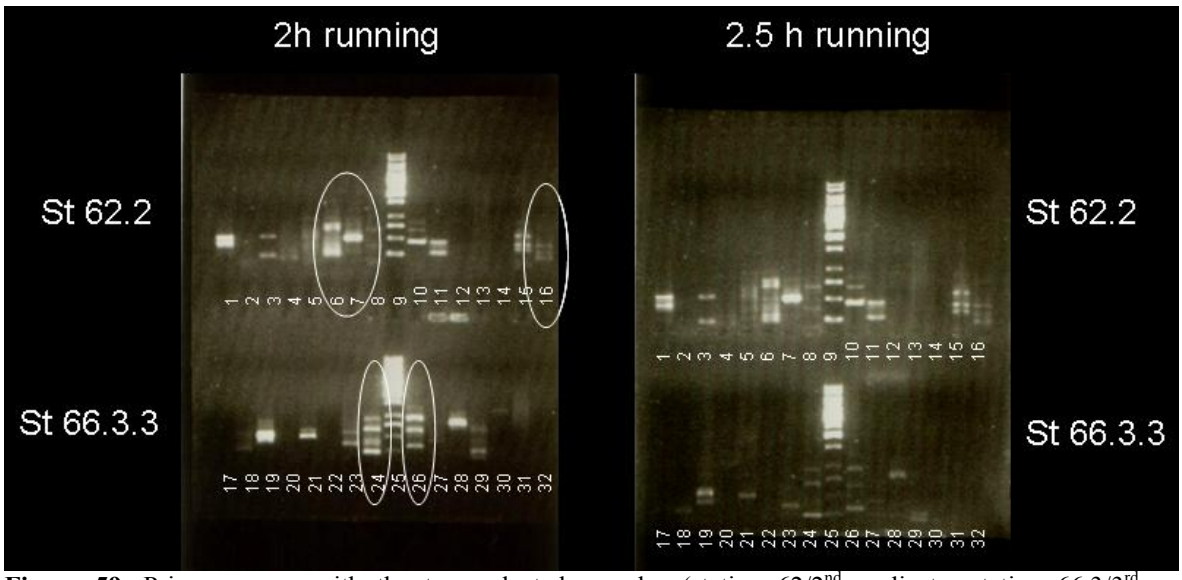

Figure 59. Primer screen with the two selected samples (station 62/2nd replicate, station 66.3/3rd replicate). Left: gel (1% agarose, SCIEPLAS) after 2 hours of electrophoresis. Right: same gel after 2.5 hours. Pools 1 to 16 correspond to analysis with sample 66.2. Pools 17 to 32 correspond to analysis with sample 66.3.3. 1:OPB-1, 2:OPB-2, 3: OPB-3, 4: OPB-4, 5: OPB-6, 6:OPB-7, 7: OPB-8, 8: OPB-10, 9: ladder, 10: OPB-11, 11: OPB-12, 12: OPB-14, 13: OPB-15, 14: OPB-16, 15:OPB-17, 16: OPB-18, 17: OPB-19, 18: control, 19: OPB-1, 20: OPB-2, 21: OPB-3, 22: OPB-4, 23: OPB-6, 24: OPB-7, 25: ladder, 26: OPB-8, 27: OPB-10, 28: OPB-11, 29: OPB-12, 30: OPB-14, 31: OPB-15, 32:OPB-16. Circles highlight pools 6 and 7, 16, and 24 and 26 with the primers OPB-7, OPB-8, and OPB-18 respectively.

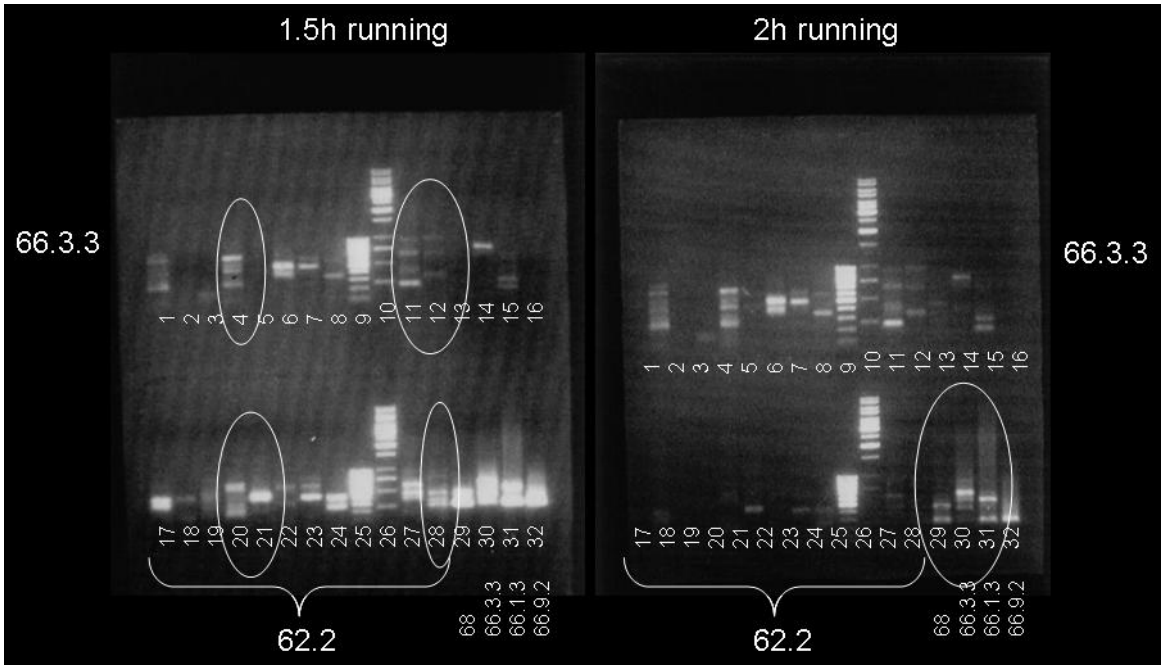

Figure 60. Test of reproducibility. Analysis with all the primers and samples from stations 66.3.3 and 62.2 confirm the results on previous gel (Figure 58) with the same samples. In this second case OPB-18 (pools 4 and 28), OPB-7 (pools 11 and 20) and OPB-8 (pools 12 and 21) give the best results in terms of bands (polymorphism) and definition. Pools 29 to 32 confirm reproducibility when using different thermocyclers. Samples from pools 29 to 31 are RAPD-PCR products for OPB-18, the same primer and samples than in figure 57, but that were run in a second thermocycler. Pools 1 to 16 are analyses with samples from station 66.3 (3rd replicate). Pools 17 to 28 are analyses with samples from station 62 (2nd replicate). 1: control, 2: OPB-16, 3: OPB-17, 4: OPB-18, 5: OPB-19, 6: OPB-1, 7: OPB-3, 8: OPB-6, 9: ladder 100bp, 10: ladder 1kbp, 11: OPB-7, 12: OPB-8, 13: OPB-10, 14: OPB-11, 15: OPB-12, 16: OPB-13, 17: OPB-1, 18: OPB-3, 19: OPB-6, 20: OPB-7, 21: OPB-8, 22: OPB-10, 23: OPB-11, 24: OPB-12, 25: ladder 100bp, 26: ladder 1kbp, 27: OPB-17, 28: OPB-18, 29: St 68 with OPB-18, 30: St 66.3.3 with OPB-18, 31: St 66.1.3 with OPB-18, 32: St 66.9.2 with OPB-18.

The three best primers (OPB7, OPB8, and OPB18) were run again with all the samples (Table 32) to prove positive results in terms of amplification of bands and polymorphism. DNA extraction was repeated in those samples that gave many negative results (see Table 32 highlight stations). However, results of RAPD-PCR with the new DNA extraction were again negative (no amplification). The only explanation for these results is that there was a problem in the preservation of those samples. Although diagnostic DNA fragments (bands) were not detected in poorly preserved samples it was possible to detect diagnostic DNA fragments (bands) that represent the population of each station in at least one of the replicates (see Table 32).

A general overview of the new results with the three best primers (samples with bands) showed again potential differences between stations because each station had different bands with the same primers (Figure 60). Although there were differences between samples of the same station (e.g. gel OPB-18, samples 66.3.4 in pool 4 and 66.3.3 in pool 5), which were most probably the result of the variability due to sexual reproduction, these differences seemed to be lower when compared with other stations (see Figure 60, samples 66.3.4 and 66.3.3 vs. samples 68 and 63).

Band-matching analysis with the software GELCOMPARE for all the samples with the primers OPB7 and OPB18 showed that a total of 105 different DNA fragments were detected, 50 with primer OPB18 and 55 with primer OPB7 (Annex 9 and 10).

Analysis of similarity between samples and between stations (cluster analysis with similarity based on Pearson correlation coefficient and UPGMA dendrogram type for single gels, and similarity based on average from experiments and UPGMA dendrogram type for cluster combining more than one gel) showed that although there were differences in the bands between stations (Figures 61 and 62), the differences were not statistically significant. Therefore, the stations correspond to the same population and may even be the same settlement event. Indeed, the clusters showed that replicates from the same station were organised in different groups, proving that the groups created by the software were not real.

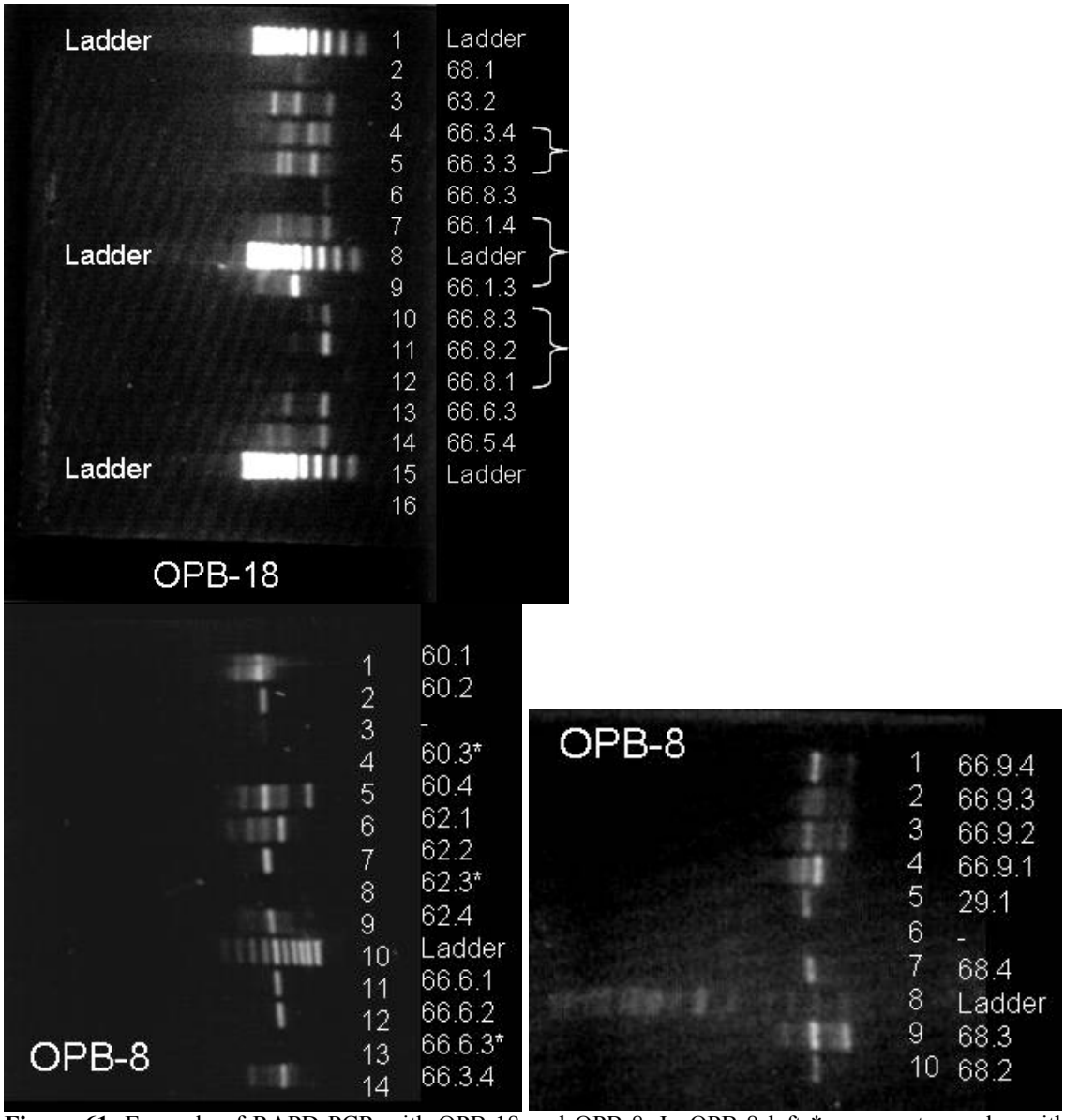

Figure 61. Example of RAPD-PCR with OPB-18 and OPB-8. In OPB-8 left * represent samples with negative results (no amplification of bands). The gels show that there are differences in the banding pattern even within the same station. Differences between samples of the same station are the result of variability due to sexual reproduction.

Table 28. Abstract of results from RAPD-PCR with OPB-18, OPB-8 and OPB-7 and all the samples (46) developed to confirm amplification of bands and polymorphism. DNA extraction was repeated in samples with many negative results (highlight). Y: positive results with more than one band. Y(1): positive results with only one band. N: negative results.

sample	OPB-18	OPB-7	OPB-8
66.1.1	Y	Y	Y(1)/Y/Y
66.1.2	Y(1)	Y	Y/Y/Y
66.1.3	Y/N/Y(1)/Y/Y(1)/Y	N	N/N/Y/N
66.1.4	Y(1)/Y/Y	Y	Y/N/Y
66.3.1	Y(1)	Y	Y/Y/Y
66.3.2	N	Y	Y/Y/Y
66.3.3	Y/Y/Y/Y	Y(1)	Y/Y/Y
66.3.4	Y/N/N/Y/Y	N	N/N
66.4.1	Y(1)	Y	Y/Y/Y
66.4.2	Y(1)	Y	Y/Y/Y
66.4.3	Y(1)/Y(1)	Y	Y/Y/Y
66.4.4	Y/Y	Y	Y/Y/Y
66.5.1	N/Y(1)/Y(1)	N/N/N	N/Y(1)/Y(1)
66.5.2	N/Y(1)/Y(1)	N/Y(1)/Y/Y	N/N/N
66.5.3	N/N/N	N/N	N/Y/Y
66.5.4	N/Y/Y	Y/Y	N/N
66.6.1	N	N/N	Y/Y(1)
66.6.2	Y(1)	N/N	Y/Y(1)
66.6.3	N/Y/Y	N/N	N/N
66.6.4	Y(1)/Y(1)	Y/Y	Y/Y
66.8.1	N/N/N	N	N
66.8.2	N/Y/Y	N	N
66.8.3	Y/N/Y(1)/Y	N	N
66.8.4	Y	Y	Y/Y
66.9.1	Y(1)	Y	N/Y
66.9.2	Y(1)	Y	Y/Y
66.9.3	Y(1)	Y	Y/Y
66.9.4	Y(1)	N/N/N	Y/Y
60.1	Y/Y(1)	Y	Y
60.2	Y(1)	N	Y(1)
60.3	N/N/N	N/N/N/N	Y/N/N/N
60.4	Y	N/N/N/N	N/Y
62.1	Y(1)	N	Y
62.2	Y	Y(1)	Y(1)
62.3	Y/N	Y(1)/Y/Y(1)	N/N
62.4	Y(1)	Y/Y	Y/Y(1)
63.1	Y(1)	N	Y/Y
63.2	N/Y/Y	N	N/N
63.3	N/N/N	N/N/N/N	N/N/N/N
63.4	N/N	Y/N/N/Y	Y/N/N/N
68.1	N/Y(1)/Y(1)	N	N
68.2	Y(1)	N	Y(1)
68.3	Y/Y/Y/Y	Y	N/Y
68.4	N/Y	Y	Y(1)/Y(1)
29,1	N/N/Y(1)/Y(1)	N/Y(1)/Y(1)	N/N/Y(1)/N/N

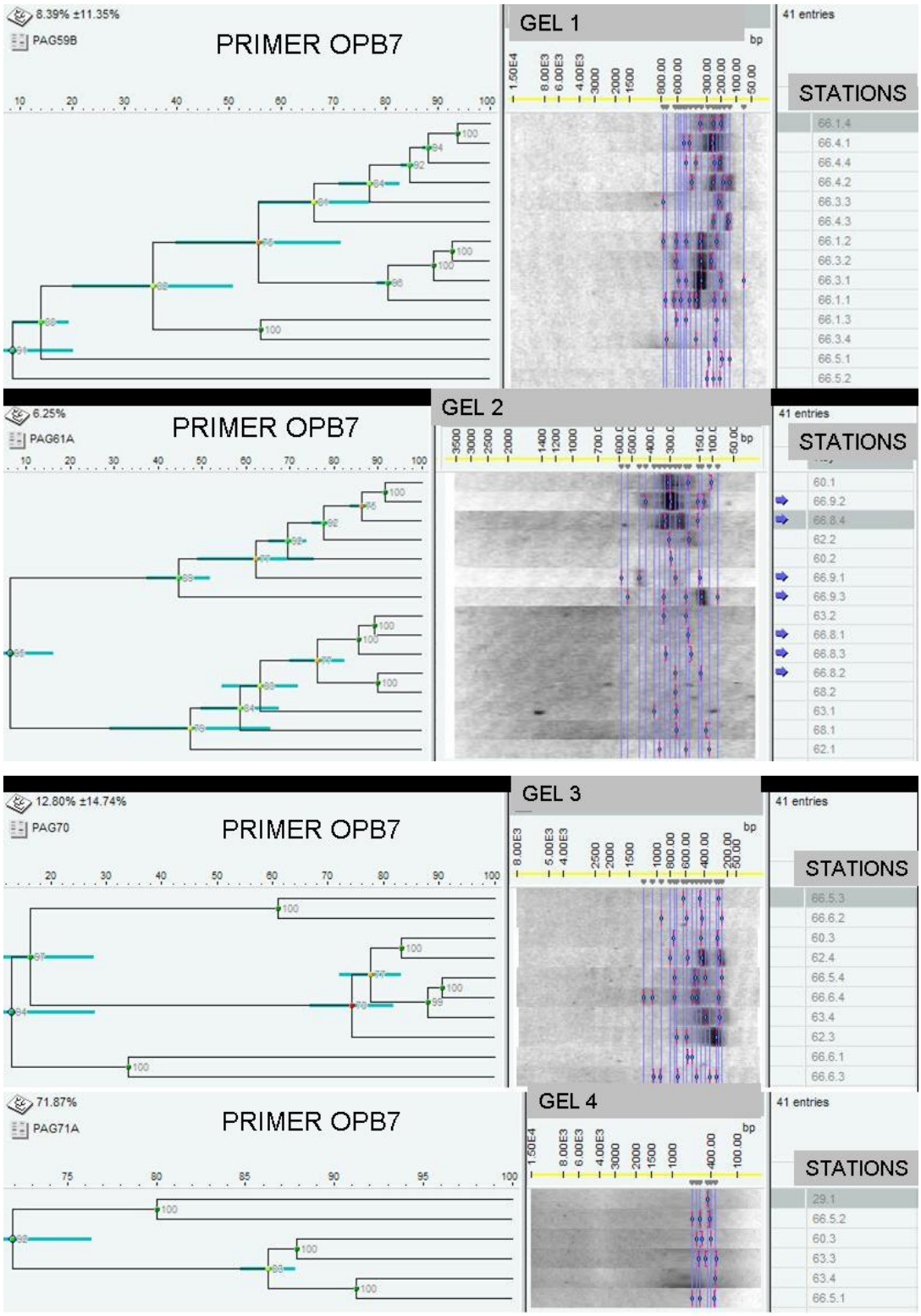

Figure 62. Results of cluster analyses (similarity based on Pearson correlation coefficient and UPGMA dendrogram type for each gel) for samples run with primer OPB7. Total samples analysed with OPB7: 41. Column stations show each of the stations and replicates (e.g. station 66.1 and replicate 4 as: 66.1.4). Each dendrogram presents in each node the error flags and the co-phenetic correlations.

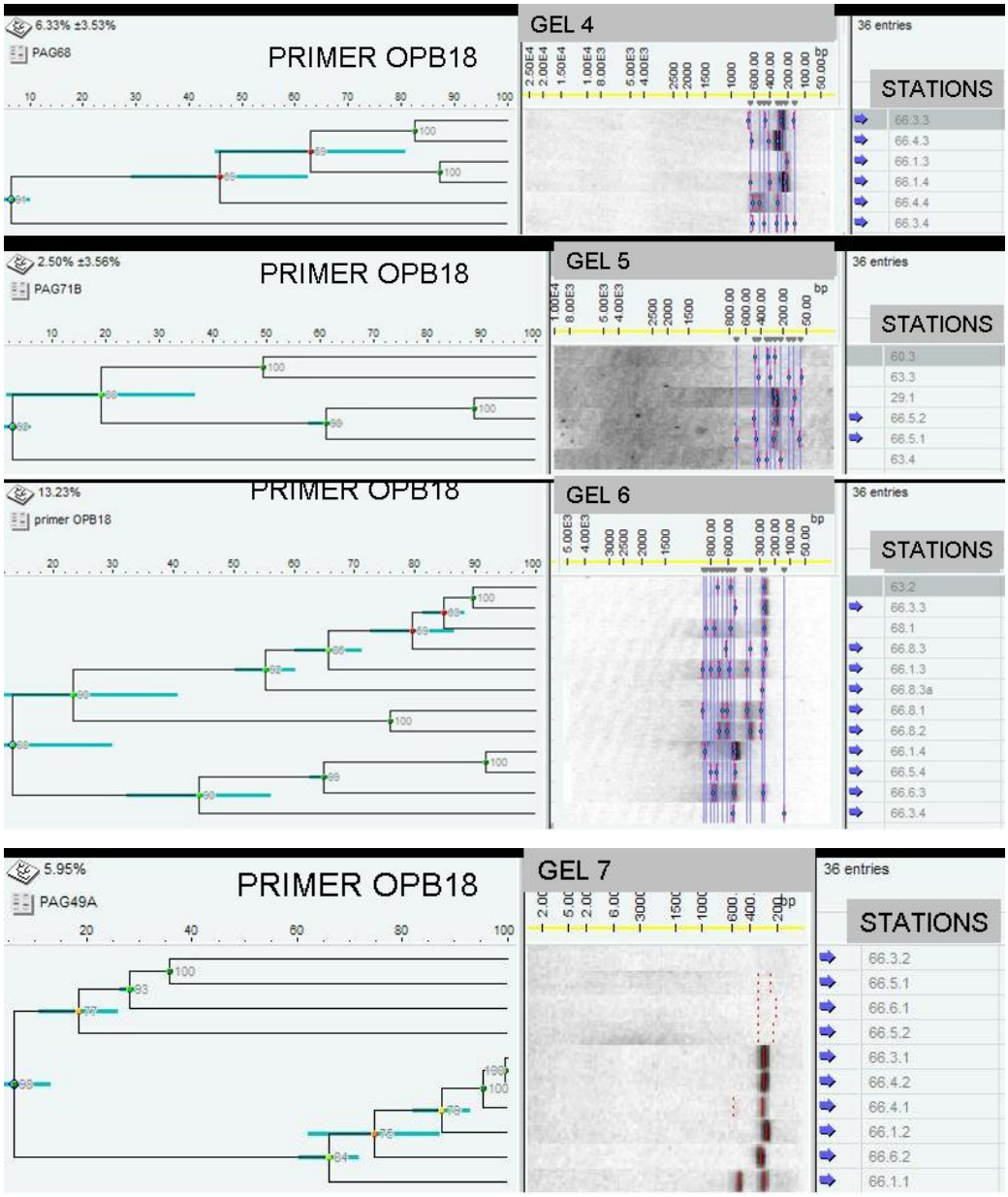

Figure 63. Results of some cluster analyses (similarity based on Pearson correlation coefficient and UPGMA dendrogram type for each gel) for samples run with primer OPB18. Total stations analyzed with OPB18: 36. Column stations show each of the stations and replicates (e.g. station 66.3 and replicate 3 as: 66.3.3). Each dendrogram presents in each node the error flags and the co-phenetic correlations.

5.6. Discussion:

5.6.1. Traditional taxonomy and ecology:

The general analysis and the PCA developed with the information of the 5 reefs in the SE of Las Perlas Archipelago and the samples collected from a coral reef environment (station 29) show that reef building and the distribution of polychaete reefs in Las Perlas Archipelago is dependent firstly on depth, a suitable substrate (topography), the potential particle size to produce the tubes or percent of granule, and wave exposure.

The ability to control abdominal growth is important so that the animal is not longer than the tube and vulnerable to predation. In other species (eg *Sabellaria aveolata* from the NE Atlantic) there is a link between the relationship of the size of the building organ, the diameter of the anterior region and the length of animal which determines the size of particles used in tube building (Gruet, 1984). Coral cover and visibility (as second variables in importance for the distribution in Las Perlas Archipelago according to the PCA) only represent the main variables for the distribution of coral reef environments. These variables are important for the polychaetes when they are not able to create their own reef because they have already settled in a reef environment. This was the case of station 29.

The values of density in the area (72.2 organisms/10 square cm) were similar to that reported by Caline *et al.* (1988) for *Sabellaria alveolata*. Volvelle (1965) and Wilson (1929) also mention that fragments of rock and shells that are captured continuously by the tentacles of each organism are glued to the substrate and added to form the cylindrical tube.

I cretus reefs provide a habitat for a diverse fauna that that in other parts of the archipelago are provided by coral reefs (see Table 28 and 30). Indeed, Hendrick and Foster-Smith (2003), in a description of the *Sabellaria spinulosa* reefs in the UK, mention the official definition of "reef" for the European Union Habitats Directive as follows: "reefs are submarine or exposed at low tide, rocky substrates and biogenic concretions, which arise from the sea floor in the sublittoral zone but may extend into the littoral zone where there is an uninterrupted zonation of plant and animal communities. These reefs generally support a zonation of benthic communities of algae and animal species including concretions, encrustations and corallogenic concretions". The animal community will use the physical and ecological habitat supplied by any type of reef in the area. In the case of Las Perlas these are the coral reefs in the North and West, and Sabellarid reefs in the South and East of the archipelago.

5.6.2. Molecular analysis:

Although the gels showed some differences in the bands between the stations the differences were not statistically significant. The interpretation of these results is that the different reefs (stations) in Las Perlas Archipelago have a similar origin. The

conclusion is that the reefs were from one population (one or more larvae or invasion of a new colony) that migrated from the south, probably Colombia or Ecuador. The population may have arrived first at San Telmo (the biggest reef), where they found good conditions for settlement and survival and from there the organisms spread to the other stations. The hypothesis about the origin of the larvae is based on the predominant current in the area, the South Equatorial Current-SEC, which transport waters from South America to Panama (see Figure 16, Glynn and Mate 1997).

To confirm the previous assumption, it is essential: 1) to collect material from other areas in the TEP (e.g. Colombia, Ecuador, Costa Rica) to run RAPD-PCR together with the samples from Las Perlas Archipelago, that would confirm the origin of the Panamanian populations. 2) To sequence some of the common bands to confirm that they are indeed the same section of DNA and not only bands with the same size. In order to sequence these common bands it is essential to acquire more specific primers. Specific primers have not been registered for Sabellarids in the GenBank, which means that the second step would include the elaboration of new primers.

5.7. Conclusions:

The distribution of polychaete reefs in Las Perlas Archipelago and the reef building itself seem to be mainly dependent on depth, a suitable substrate (topography), the potential particle size to produce the tubes or percent of granule, and wave exposure. Other factors not measured during this work such as length of prostomial tentacles and the ability to control abdominal growth may also be paramount factors.

I cretus reefs in the south part of the Archipelago may be offering physical and ecological space to a large diversity of associated fauna that in other parts of the Archipelago are provided by the coral reefs.

The polychaete reefs in Las Perlas Archipelago correspond to one population (group of larvae or invasion of a colony) that migrated from the south, probably Colombia or Ecuador.

PART 4. GENERAL CONCLUSIONS

From the component related to the taxonomy and ecology of vermetid from Las Perlas (chapter 8) it was obtained the folloing conclusions:

It is described a new morphotype of *E. tulipus* associated to coral reef environments in Las Perlas Archipelago (Pacific of Panama). Observations in other areas show that its distribution could include the whole TEP.

The specie showed an annual growth rate of 24mm with big variations among stations. These variations seem to be the response of the specie to the surrounded environment, especially to the competition with the coral species that create the reef frame.

It was possible to identify annual, monthly (spring tide bands) and daily growth bands. The monthly bands seem to be organized in two different groups: small and compact spring tide bands, related to the upwelling months, and bigger spring tide bands produced by the mollusk during the rest of the year. The differences in the size fo the annual growth bands may be result of environmental factors that a affect a bigger scale such as El Niño-ENSO.

In terms of microhabitat, the strong relation between the presence of vermetids and healthy branched coral reefs just express the requirements of the mollusc for environments that bring a good protection against predators and a direct surface to the water column so they can access for food. However the specie is more flexible when it comes to the general reef environment. In this last case, the coral cover can be lower and the frame could include other benthic components apart from the branched corals. The only requirement is to be a proper reef frame, with a minimal vertical growth so to aloud a good protection to the vermetid.

The distribution pattern of the species in the reef environments of Las Perlas Archipelago is, according to the statistical analysis, the result of a random offer of clean space for settlement and a tendency to solitary life. In this way, the general requirements of the species are the same no mater the environment, rocky or coral reef areas.

PART 4. General conclusions

The analysis of the vermetids shells and secondary information through sclerochronology and time series analysis (chapter 9) evidenced the following results:

The shells from Las Perlas Archipelago (Panama) showed a longitudinal annual growth rate of 24mm/year and detectable annual, monthly and daily bands.

X-ray diffraction and SEM analysis showed aragonite as the main shell component.

Concentrations of oxygen isotopes in water showed similar results to those reported previously for the area.

Comparisons between shells and water oxygen isotopes showed that accumulation of oxygen isotopes in shells are not in isotopic equilibrium with the sea water but are nearer of the isotopic equilibrium than corals.

Analysis of trace elements (Sr, Mg, and Ba) and oxygen isotopes under different methodologies showed that there is detectable accumulation of the elements.

Analysis of trace elements showed a positive relation between the Mg signal and SST and a negative relation between Sr signal and SST. There seems to be a negative relation between the Ba signal and salinity but it is necessary to perform more analysis in order to confirm this relation.

The relation between the trace elements and the records for SST and salinity are in general agreement with previous literature for other marine invertebrates.

The results also showed a negative relation between $\delta^{18}O$ and SST that is in general agreement with previous literature for other marine invertebrates.

Comparisons between methodologies for trace elements showed ICP-OES as the best methodology to analyze annual growth bands in the vermetid, with a higher accuracy and lower cost. When more detail and/or minimal amount of sample are needed IMF is the most practical methodology. Besides, it gives the option to compare the results of the analysis with the band pattern from where they were collected (pictures of samples analyzed).

Comparisons between methodologies for oxygen isotopes showed that MS was good to analyze general growth band patterns, but IMF was better to obtain more detail and been able to compare the results of the analysis with the band pattern from where they were collected.

On the other hand, ecological and molecular analyis of the species Idanthyrsus cretus (chapter 10) gave the following results:

The distribution of polychaete reefs in Las Perlas Archipelago and the reef building itself seem to be mainly dependent on depth, a suitable substrate (topography), the potential particle size to produce the tubes or percent of granule, and wave exposure. Other factors not measured during this work such as length of prostomial tentacles and the ability to control abdominal growth may also be paramount factors.

I cretus reefs in the south part of the archipelago may be offering physical and ecological space to a big diversity of associated fauna that in other parts of the archipelago are provided by the coral reefs.

The polychaete reefs in Las Perlas Archipelago correspond to one population (group of larvae or invasion of a colony) that migrated from the south, probably Colombia or Ecuador.

APPENDICES

Annex 1. Vermetid samples from Las Perlas Archipelago (Panama) for molecular analysis (alcohol 99%)

SP	DESCRIPTION	Station	ISLAND	# TUBES	TOTAL# organisms	DEPTH	BOTTOM TYPE
E. tulipus sp1	Within massive coral, no snorkel, sample from one tube (2004) sent to Tim Rawlings on 05-11-05 (good results).	**18** 8° 35.537'N 79°1.166'W	Chapera	2	7	2-7M	Coralline area (medium sand)
E. tulipus sp1	Tube with snorkel and brown operculum (NO SAMPLES SENT)	29 8° 27'36.48"N 78°51'8.29''W	San Pedro	3	6	2-7M	Coralline area (medium sand)
E. tulipus sp1	Tube with snorkel and brown operculum (NO SAMPLE SENT)	12 8° 38'02"N 79°02'09"W	contadora	1	2	2-7M	Coralline area (medium sand)
E. tulipus sp1	Tube with snorkel and brown operculum, one sample from 2004 and other from 2005 sent to Tim Rawlings on 05-11-05. One sample with good results	**55** 8° 25'13"N 79°6'41"W	Pedro Gonzáles	3	13	4M	Coralline area (medium sand)
E. tulipus sp1	With operculum, shell without snorkel, in rocky area (1 smaller organism) (2 SAMPLES SENT ON 3-05-06)	**103** 8°54'26.4"N 79°31'05.5"W	flamencos	2	2	7M	Rocky area (fine sand)
E. tulipus Sp 2	Tubes similar to those of E. *tulipus*. Organism without operculum. Anterior part is white when alive (NO SAMPLE SENT)	12 8°38'02"N 79°02'09"W	Contadora	1	1	2-7M	Coralline area (medium sand)
E. tulipus Sp 2	Tubes similar to those of *E. tulipus*. Organism without operculum. Anterior part is white when alive (2 SAMPLES SENT ON 3-05-06)	**13** 8°33'51.6"N 79°01'13.1"W	Mogo-Mogo	2	5	2-7M	Coralline area (medium sand)
E. tulipus Sp 2	Tubes similar to those of *E. tulipus*. Organism without operculum. Anterior part is white when alive 2 samples sent to Tim Rawlings on 05-11-05 (no results)	**55** 8° 25'13"N 79°6'41"W	Pedro Gonzáles	2	3	4M	Coralline area (medium sand)
E. tulipus sp3 (*Vermetus erectus*)	Tubes with operculum but without snorkel and smaller, rocky area. 1 sample sent to Tim Rawlings on 05-11-05 (good results)	**101** 8° 48'20.4"N 79°33'12.9"W	Taboga	2	2	7M	Rocky area (fine sand)

E. tulipus sp 3/4	Animal with operculum concave and not spiral but with 3 concentric circles, tube without snorkel, smaller, rocky area. Similar to original sp3 (identified as *Vermetus erectus*). NO SAMPLE SENT	102 8°48.1'N 79°31.2'W	Taboguilla	2	2	7M	Rocky area (fine sand)
E. tulipus Sp 3/4	Animal with operculum concave and not spiral but with 3 concentric circles, tube without snorkel, smaller, rocky area. Similar to original sp3 (identified as *Vermetus erectus*). 2 SAMPLES SENT ON 3-05-06	**103** 8°54'26.4"N 79°31'05.5"W	Flamencos	2	2	7M	Rocky area (fine sand)
E. tulipus sp5	Similar to sp ¾ but with doubts about similarity (same type of operculum, with 3 concentric circles). 1 HOLE SAMPLE SENT ON 3-05-06 (all animal)	**102** 8°48.1'N 79°31.2'W	Taboguilla	2 including shell for taxonomic comparisons	2 (molecular analysis and taxonomic comparisons)	7M	Rocky area (fine sand)
E. tulipus sp6	Tube without snorkel, animal without operculum (Similar to sp 2 but without snorkel), smaller, colour anterior part is black/white with a tiger pattern. 1 HOLE SAMPLE SENT ON 3-05-06 (all animal)	**102** 8°48.1'N 79°31.2'W	Taboguilla	2 including shell for taxonomic comparisons	2 (molecular analysis and taxonomic comparisons)	7M	Rocky area (fine sand)

Stations **XX**: samples sent to Tim Rawlings: (tissue from mollusc foot).

Annex 2. Results of PCA that identify the main components responsible of the microhabitat observed for *E. tulipus* in Las Perlas Archipelago.

	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue	2,316	1,516	1,086	0,569	0,321	0,192
Proportion	0,386	0,253	0,181	0,095	0,053	0,032
Cumulative	0,386	0,639	0,820	0,915	0,968	1,000
COEFFICIENTS						
variable	PC1	PC2	PC3	PC4	PC5	PC6
DEPTH	-0,440	-0,457	-0,226	0,418	-0,273	0,545
CORALSPP	0,591	0,024	0,233	0,083	0,327	0,694
CORALCOVER	0,389	-0,510	-0,175	0,526	0,281	-0,450
CORALDENSITY	0,115	0,671	-0,078	0,666	-0,293	-0,036
SLOPE	0,540	-0,190	-0,226	-0,256	-0,746	0,005
MOLUSCSTATUS	0,042	0,210	-0,898	-0,180	0,313	0,133

Annex 3. Results of PCA for the main factors that describe the reef complexity observed in Las Perlas Archipelago.

	PC1	PC2	PC3
Eigenvalue	1,370	1,003	0,627
Proportion	0,457	0,334	0,209
Cumulative	0,457	0,791	1,000
COEFFICIENTS			
variable	PC1	PC2	PC3
thickness	-0,471	0,744	-0,474
Number vermetids	0,525	0,669	0,526
coral density	0,709	-0,001	-0,706

Annex 4. Results of PCA for the main factors that explain the current structure of coral reefs in Las Perlas Archipelago.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Eigenvalue	2,348	1,664	1,005	0,822	0,813	0,348	0,0
Proportion	0,335	0,238	0,144	0,117	0,116	0,050	0,0
Cumulative	0,335	0,573	0,717	0,834	0,950	1,000	1,0
COEFFICIENTS							
variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Topography	0,441	-0,385	0,038	-0,222	-0,306	0,716	0,0
Depth	-0,235	-0,336	-0,539	-0,532	-0,383	-0,335	0,0
mumber coral spp	-0,246	-0,127	0,721	0,020	-0,601	-0,205	0,0
life coral cover	-0,601	-0,164	0,074	-0,105	0,247	0,351	-0,6
dead coral cover	0,158	0,639	-0,231	0,074	-0,498	0,069	-0,5
others cover	0,529	-0,377	0,119	0,051	0,161	-0,450	-0,5
number fish spp	-0,154	-0,381	-0,340	0,805	-0,254	0,049	0,0

Annex 5. Preliminary results for δ18O and δ13C isotopes analysis from samples PAN-04-55-1 and PAN-04-55-2. *Mean value for MAB2B (analysis between December 2003 and March 2005 at Edinburgh University laboratories) is -2.475 PDB for $\delta^{18}O$ and 2.424 PDB for $d^{13}C$. ** Mean value for COR1D (analysis between December 2003 and March 2005 at Edinburgh University laboratories) is -4.958 PDB for $\delta^{18}O$ and -0.688 PDB for $d^{13}C$. A= values of $d^{13}C$ for *Tridacna* sp between -0.815 and 2.014 PDB. B= values of $\delta^{18}O$ for *Tridacna* sp between -0.738 and -3.563 PDB.

SHELL PAN-04-55-1							
PORT/DATE	**SAMPLE ID**	**µg SAMPLE**	**d13C (‰PDB)**	**d18O (‰PDB)**	**OBSERV.**	**RESIN**	**EPIFAUNA**
8 (14-03-05)	V8	0,23	2,154	-2,612	Oldest band		
7 (14-03-05)	V7	0,15	1,913	-2,776			
6 (14-03-05)	V6	0,12	1,832	-2,747			
5 (14-03-05)	V5	0,11	1,720	-2,795			
4 (14-03-05)	V4	0,13	1,829	-2,767			
35 (10-03-05)	V3	0,20	2,160	-2,294			
34(10-03-05)	V2	0,24	2,148	-2,231			
33 (10-03-05)	V1	0,21	2,001	-2,264	Youngest band		
STANDARDS PAN-04-55-1							
21 (14-03-05)	COR1D	0,190	-0,881	-5,187	Final standards		
20 (14-03-05)	MAB2B	0,150	2,463	-2,470			
19 (14-03-5)	MAB2B	0,260	2,422	-2,483			
38 (10-03-05)	COR1D	0,170	-0,791	-5,212			
37 (10-03-05)	MAB2B	0,250	2,565	-2,416			
36 (10-03-05)	MAB2B	0,100	2,354	-2,568			
18 TO 9 (14-03-05)	*Tridacna* spp		A	B			
8 TO 4 (14-03-05)	**PAN-04-55-1**						
3 (14-03-05)	COR1D	0,190	-0,777	-5,067	First standards		
2 (14-03-05)	MAB2B	0,260	2,454	-2,470			
1 (14-03-05)	MAB2B	0,120	2,343	-2,608			
21 (10-03-05)	COR1D	0,300	-0,539	-4,846			
20 (10-03-05)	MAB2B	0,240	2,290	-2,664			
19 (10-03-05)	MAB2B	0,250	1,922	-3,033	resin from previous sample		

SHELL PAN-04-55-2							
PORT/DATE	**SAMPLE ID**	**µg SAMPLE**	**d13C (‰PDB)**	**d18O (‰PDB)**	**OBSERV.**	**RESIN**	**EPIFAUNA**
17 (10-03-05)	TV14	0,25	-2,111	-1,498	Oldest band		X
16 (10-03-05)	TV13	0,12	-0,891	-1,284			X
15 (10-03-05)	TV12	0,23	-0,097	-1,301			X
14 (10-03-05)	TV11	0,30	-0,320	-1,796		X?	X?
13 (10-03-05)	TV10	0,14	-0,162	-1,748			
12 (10-03-05)	TV9	0,13	-0,174	-2,067			
11 (10-03-05)	TV8	0,26	-0,493	-2,135			
10 (10-03-05)	TV7	0,24	0,718	-1,171			
9 (10-03-05)	TV6	0,27	-0,067	-2,121			
8 (10-03-05)	TV5	0,25	-0,025	-2,404			
7 (10-03-05)	TV4	0,21	0,141	-2,562			
6 (10-03-05)	TV3	0,21	0,226	-2,641			
5 (10-03-05)	TV2	0,19	-0,105	-2,712			
4 (10-03-05)	TV1	0,13	-0,475	-2,728	Youngest band	X?	
STANDARDS PAN-04-55-2							
21 (10-03-05)	COR1D	0,300	-0,539	-4,846	Final standards		
20 (10-03-05)	MAB2B	0,240	2,290	-2,664			
19 (10-03-05)	MAB2B	0,250	1,922	-3,033			
18 (10-03-05)	PAN-55-3						
4 TO 17 (10-03-05)	PAN-04-55-2						
3 (10-03-05)	COR1D	0,20	-0,758	-5,143	First standards		
2 (10-03-05)	MAB2B	0,25	2,582	-2,276			
1 (10-03-05)	MAB2B	0,18	2,476	-2,443			

Annex 6. Results of ICP-OES analysis for Mg and Sr in weight percent (percent of each element with respect to the total weight of the sample) and in ppm (mmol element/mol Ca). MAD91-1 to MAD91-5 correspond to coral standards.

	Weight % data						Mole ratio data			
Sample ID	Ca wt%	% RSD	Mg wt%	% RSD	Sr wt%	%RSD	mmolMg/ molCa	% RSD Mg/Ca	mmolSr/ molCa	% RSD Sr/Ca
1	39,66	0,19	0,0171	0,134	0,1594	0,113	0,7113	0,23	1,8380	0,22
2	38,87	0,18	0,0138	0,208	0,1468	0,067	0,5837	0,27	1,7275	0,19
3	39,17	0,16	0,0169	0,138	0,1449	0,042	0,7114	0,21	1,6921	0,16
4	39,62	0,17	0,0127	0,082	0,1518	0,153	0,5290	0,18	1,7531	0,23
5	38,98	0,04	0,0102	0,104	0,1508	0,179	0,4328	0,11	1,7697	0,18
6	39,79	0,03	0,0095	0,181	0,1642	0,183	0,3933	0,18	1,8879	0,19
7	38,66	0,04	0,0105	0,170	0,1828	0,120	0,4479	0,17	2,1628	0,13
8	39,15	0,06	0,0076	0,264	0,1930	0,057	0,3184	0,27	2,2544	0,09
9	39,36	0,03	0,0097	0,074	0,2108	0,048	0,4056	0,08	2,4504	0,06
10	39,31	0,24	0,0094	0,512	0,2034	0,106	0,3947	0,56	2,3667	0,26
11	39,47	0,12	0,0099	0,228	0,2117	0,152	0,4153	0,26	2,4530	0,20
12	39,34	0,11	0,0095	0,095	0,2054	0,026	0,3986	0,15	2,3879	0,12
13	39,76	0,02	0,0148	0,225	0,2150	0,025	0,6118	0,23	2,4741	0,03
14	39,57	0,14	0,0148	0,062	0,2038	0,114	0,6155	0,15	2,3553	0,18
15	39,90	0,13	0,0090	0,513	0,2056	0,173	0,3724	0,53	2,3574	0,22
16	39,84	0,09	0,0112	0,316	0,1941	0,099	0,4619	0,33	2,2290	0,13
17	36,11	0,09	0,0088	0,404	0,1634	0,141	0,4037	0,41	2,0692	0,17
18	38,33	0,16	0,0109	0,123	0,1807	0,054	0,4694	0,21	2,1559	0,17
19	39,85	0,10	0,0168	0,338	0,2037	0,077	0,6935	0,35	2,3382	0,13
20	39,63	0,09	0,0148	0,683	0,1981	0,109	0,6166	0,69	2,2861	0,14
21	38,16	0,06	0,0122	0,152	0,1858	0,039	0,5254	0,16	2,2267	0,07
22	35,89	0,15	0,0203	0,236	0,1882	0,204	0,9340	0,28	2,3982	0,26
23	35,68	0,11	0,0125	0,205	0,1813	0,068	0,5759	0,23	2,3244	0,13
24	39,05	0,16	0,0155	0,128	0,2049	0,166	0,6533	0,21	2,3995	0,23
25	39,05	0,03	0,0115	0,247	0,2052	0,041	0,4865	0,25	2,4041	0,05
26	39,19	0,03	0,0129	0,546	0,1980	0,033	0,5415	0,55	2,3111	0,04

27	39,25	0,04	0,0116	0,249	0,1951	0,073	0,4873	0,25	2,2738	0,08
28	40,35	0,21	0,0163	0,067	0,2050	0,203	0,6665	0,22	2,3243	0,29
29	39,25	0,04	0,0142	0,309	0,1998	0,031	0,5962	0,31	2,3285	0,05
30	39,24	0,06	0,0129	0,148	0,1968	0,100	0,5412	0,16	2,2942	0,12
31	39,24	0,05	0,0138	0,137	0,1940	0,088	0,5807	0,15	2,2613	0,10
32	39,46	0,04	0,0144	0,209	0,1891	0,070	0,6027	0,21	2,1917	0,08
33	39,23	0,03	0,0295	0,024	0,1916	0,019	1,2397	0,03	2,2336	0,03
34	38,19	0,07	0,0206	0,244	0,1884	0,022	0,8907	0,25	2,2564	0,08
35	35,40	0,16	0,0216	0,140	0,1767	0,289	1,0057	0,21	2,2827	0,33
36	38,73	0,21	0,0275	0,092	0,1902	0,102	1,1693	0,23	2,2466	0,23
MAD91-1	38,82	0,14	0,1068	0,090	0,7341	0,056	4,5361	0,17	8,6495	0,15
MAD91-2	38,63	0,07	0,1060	0,069	0,7325	0,053	4,5238	0,10	8,6720	0,09
MAD91-3	34,79	0,02	0,0958	0,037	0,6595	0,035	4,5426	0,04	8,6724	0,04
MAD91-4	37,03	0,12	0,1016	0,008	0,7022	0,078	4,5243	0,12	8,6741	0,14
MAD91-5	39,62	0,11	0,1099	0,079	0,7504	0,081	4,5737	0,13	8,6635	0,13
Blank 1	-0,02	0,41	0,0007	6,474	-0,0004	36,770				
Blank 2	-0,02	38,33	0,0008	3,953	-0,0005	11,853				
Blank 3	0,04	21,47	0,0009	3,610	-0,0001	130,070				
Blank 4	0,00	2567,87	0,0006	7,096	-0,0002	27,825				
Blank 5	-0,01	51,16	0,0006	11,184	-0,0004	18,942				

Annex 7. Results of non parametric Kendall rank correlation coefficient-T test. Sample weight sorted from smaller to larger values. Mg and Sr are sorted according to sample weight. A1 and B1 are categories for Mg. A2 and B2 are categories for Sr. S1=Σ(A1-B1), S2= Σ(A2-B2). N1=N2=36 samples. K1= T test for correlation between sample weight and Mg. K2= T test for correlation between sample weight and Sr. Z1= calculated probability for K1. Z2= calculated probability for K2. P1= Tabulated probability for K1 (α= 0.05). P2= Tabulated probability for K2 (α= 0.05). Z1>P1 and Z1>2.58= highly significant correlation between sample weight and Mg. Z2<P2 and Z2<1.96= no correlation between sample weight and Sr.

sample weight (mg)	Mg	A1	B1	Sr	A2	B2
3,473	0,3184	35	0	2,2544	20	15
4,106	0,3947	32	2	2,3667	6	28
4,543	0,4153	28	5	2,4530	0	33
4,823	0,7114	6	26	1,6921	32	0
4,829	0,3986	29	2	2,3879	4	27
4,837	0,3933	29	1	1,8879	25	5
4,846	0,4056	27	2	2,4504	0	29
4,867	0,5290	19	9	1,7531	26	2
4,878	0,6155	11	16	2,3553	4	23
4,935	0,3724	26	0	2,3574	3	23
4,992	0,4479	23	3	2,1628	19	6
4,992	0,6166	10	15	2,2861	9	15
5,004	0,4619	21	3	2,2290	15	8
5,014	0,7114	5	18	1,6921	22	0
5,056	0,6935	6	16	2,3382	3	18
5,057	0,7113	5	16	1,8380	18	2
5,15	0,9340	3	17	2,3982	2	17
5,18	0,6665	4	15	2,3243	4	14
5,363	0,4694	15	3	2,1559	14	3
5,455	0,4865	14	2	2,4041	0	16
5,478	0,4037	15	0	2,0692	13	2
5,536	0,5837	7	7	1,7275	14	0
5,752	0,5415	9	4	2,3111	3	10
5,884	1,0057	2	10	2,2827	4	8
5,958	1,1693	1	10	2,2466	7	4
5,974	0,4328	10	0	1,7697	10	0
6,056	0,4873	9	0	2,2738	4	5
6,513	0,5759	6	2	2,3244	2	6
6,715	0,5254	7	0	2,2267	6	1
6,931	0,6027	3	3	2,1917	6	0
7,066	0,5962	3	2	2,3285	1	4
7,224	0,5412	4	0	2,2942	1	3
7,285	0,6533	2	1	2,3995	0	3
7,453	0,8907	1	1	2,2564	1	1
7,576	0,5807	1	0	2,2613	1	0
7,731	1,2397			2,2336		
CORRELATION WEIGHT-MG						
S1	N1	K1	Z1	P1		
217	36	0,3444	2,9557	0.0016		
CORRELATION WEIGHT-SR						
S2	N2	K2	Z2	P2		
-32	36	-0,0508	-0,4359	>0.5		

Annex 8. Type of sediments in areas around the stations (values for granule and components in percentage-%).

Nearest polychaete station	**Station for sediment**	**Sediment Type**	Granule	Very coarse Sand	Coarse Sand	Medium Sand	Fine Sand	Very fine Sand	Silt-Clay
66	22	sand	4.6	17.4	24.9	39.5	13.0	0.2	0.5
68,60,62,63	23	sand + shells	1.3	1.1	1.0	4.2	58.4	22.6	11.5
68,60,62,63	36	mud + sand	4.1	1.6	1.3	1.7	13.0	60.5	17.8
29	61	Mud	0.0	0.2	0.1	0.0	8.7	36.3	54.7
29	62	Mud	0.2	0.0	0.3	0.9	7.3	38.3	52.9
29	77	mud	0.6	0.2	0.1	0.3	1.1	17.9	79.9
29	78	Mud	0.5	1.0	0.6	0.2	1.1	10.3	86.2

Annex 9. Results of PCA to identify the main variables responsible of the reef distribution in Las Perlas Archipelago.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12
Eigenvalue	7,392	4,608	0,000	0,000	0,000	0,000	0,000	0,000	0,000	-0,000	-0,000	-0,000
Proportion	0,616	0,384	0,000	0,000	0,000	0,000	0,000	0,000	0,000	-0,000	-0,000	-0,000
Cumulative	0,616	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000
COEFFICIENTS												
	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12
DEPTH	0,366	-0,046	-0,185	0,116	-0,228	0,011	-0,280	-0,605	-0,540	-0,069	-0,137	0,067
Granule	0,368	-0,006	-0,311	-0,232	0,043	-0,414	0,320	0,413	-0,299	0,287	-0,253	0,181
Very coarse Sand	0,274	-0,311	0,223	-0,064	-0,443	-0,339	-0,316	0,332	0,037	-0,329	0,379	-0,025
Coarse Sand	0,268	-0,319	-0,040	-0,353	-0,301	0,390	0,443	-0,253	0,271	0,187	0,244	0,173
Medium Sand	0,276	-0,308	0,028	0,191	-0,114	0,304	-0,275	0,227	0,238	0,281	-0,529	-0,378
Fine Sand	0,015	0,465	-0,163	-0,294	-0,292	-0,376	-0,243	-0,266	0,464	0,263	-0,058	-0,158
Very fine Sand	-0,141	0,430	-0,050	-0,349	-0,386	0,512	-0,146	0,358	-0,319	-0,084	-0,044	0,015
Silt-Clay	-0,347	-0,154	-0,133	0,192	-0,255	-0,090	0,115	0,014	-0,322	0,502	0,375	-0,467
wave exposure	0,363	0,074	-0,216	-0,204	0,554	0,203	-0,365	0,074	-0,017	0,162	0,481	-0,173
visibility (m)	-0,248	-0,344	0,242	-0,691	0,197	-0,126	-0,070	-0,170	-0,196	-0,090	-0,233	-0,309
coral cover	-0,281	-0,300	-0,814	-0,037	-0,039	0,022	-0,092	0,052	0,167	-0,353	-0,043	-0,008
topography	0,308	0,255	-0,055	0,054	-0,003	0,000	0,456	-0,036	0,000	-0,452	0,000	-0,649

Annex 10. Bandmatching ananlysis with the software GELCOMPARE for all the samples with primer OPB7. 1: confirmed band. ?: uncertain band.

Station/rep	GEL1:758.55bp	GEL1:713.77bp	GEL1:613.99bp	GEL1:582.47bp	GEL1:549.89bp	GEL1:521.97bp	GEL1:488.52bp	GEL1:447.67bp	GEL1:389.39bp	GEL1:346.13bp	GEL1:291.08bp	GEL1:252.85bp	GEL1:229.02bp	GEL1:205.13bp	GEL1:183.37bp	GEL1:141.95bp	GEL1:72.67bp	GEL2:585.86bp	GEL2:533.54bp	GEL2:457.69bp	GEL2:422.52bp	GEL2:377.01bp	GEL2:354.37bp	GEL2:328.78bp	GEL2:304.53bp	GEL2:269.66bp	GEL2:248.93bp	GEL2:220.34bp
29.1																												
60.1																									1			
60.2																									?			
60.3																												
62.1																							?					?
62.2																									1			
62.3																												
62.4																												
63.1																						?				?		
63.2																								?				?
63.3																												
63.4																												
66.1.1		?	1		1			?	1			1			1													
66.1.2	1		1				1			1			1															
66.1.3			?				?						?															
66.1.4										1		1		1														
66.3.1				?			?			1				1			?											
66.3.2			1							1		1																
66.3.3	?													1														
66.3.4		?							?				?															
66.4.1						1		1				1																
66.4.2								1				1			1	1												
66.4.3												1				1												

66.4.4							1		1			1		1														
66.5.1											?			?		?												
66.5.2											?	?		?														
66.5.3																												
66.5.4																												
66.6.1																												
66.6.2																												
66.6.3																												
66.6.4																												
66.8.1																												
66.8.2																									?			
66.8.3																								?				
66.8.4																								1			1	
66.9.1																		?		1						1		
66.9.2																					1				1			
66.9.3																			?					1				1
68.1																										?		
68.2																										?		

Annex 10. Continuation. Bandmatching ananlysis with the software GELCOMPARE for all the samples with primer OPB7.
1: confirmed band. ?: uncertain band.

Station/rep	GEL2:202.08bp	GEL2:156.30bp	GEL2:139.55bp	GEL2:112.04bp	GEL2:83.54bp	GEL3:1211bp	GEL3:1053bp	GEL3:931bp	GEL3:806.94bp	GEL3:740.66bp	GEL3:698.40bp	GEL3:631.53bp	GEL3:589.72bp	GEL3:529.55bp	GEL3:486.93bp	GEL3:433.32bp	GEL3:398.45bp	GEL3:358.03bp	GEL3:293.73bp	GEL3:275.16bp	GEL3:256.63bp	GEL4:749.46bp	GEL4:720.88bp	GEL4:682.50bp	GEL4:479.46bp	GEL4:380.14bp	GEL4:302.57bp
29.1																									1		
60.1	1			?																							
60.2																											
60.3										?						1				1			?	?		?	
62.1				?																							
62.2	1																										
62.3											1		1						1								
62.4									?				1			1				1							
63.1																											
63.2																											
63.3																								?	?		?
63.4																	1			1							?
66.1.1																											
66.1.2																											
66.1.3																											
66.1.4																											
66.3.1																											
66.3.2																											
66.3.3																											
66.3.4																											
66.4.1																											
66.4.2																											
66.4.3																											
66.4.4																											

66.5.1																						?		?			?
66.5.2																						?		1	1		
66.5.3												?				?				?							
66.5.4										1					1		1				1						
66.6.1													?	?													
66.6.2								?					?			?					?						
66.6.3							?	?			?				?			?	?								
66.6.4						1	?			1				?	1					1							
66.8.1	?																										
66.8.2			?																								
66.8.3	?																										
66.8.4		?																									
66.9.1		?																									
66.9.2		1	1																								
66.9.3			1		?																						
68.1				?																							
68.2																											

Annex 11. Bandmatching ananlysis with the software GELCOMPARE for all the samples with the primer OPB18. 1: confirmed band. ?: uncertain band.

Station/rep	GEL6:576.92bp	GEL6:268.06bp	GEL1:867.20bp	GEL1:651.14bp	GEL1:499.87bp	GEL4:260.98bp	GEL1:266.46bp	GEL4:649.99bp	GEL6:756.97bp	GEL1:443.34bp	GEL4:519.66bp	GEL1:365.60bp	GEL4:303.72bp	GEL4:217.25bp	GEL6:936bp	GEL6:524.76bp	GEL1:241.82bp	GEL4:402.44bp	GEL3:426.04bp	GEL4:451.63bp	GEL4:151.05bp	GEL5:463.76bp	GEL5:303.97bp	GEL5:219.21bp	GEL5:144.59bp
29.1																									
60.3																			?			?			
60.4																									
62.3																									
62.4																									
63.2	1	1																							
63.3																							?		?
63.4																								?	
66.1.1																									
66.1.2																									
66.1.3	1	1												1	?										
66.1.4								1						1	1	1	1	1							
66.3.1																									
66.3.2																									
66.3.3		1	1	1	1	1	1	1								1				?	?				
66.3.4							1	?				1	?	?		1				?	?				
66.4.1																									
66.4.2																									
66.4.3								?					1				1	1							
66.4.4								?		1	1	1	1				1								
66.5.1																						?			
66.5.2																						?			
66.5.3																									
66.5.4																1									
66.6.1																									
66.6.2																									

66.6.3		1							1							1									
66.6.4																									
66.8.1															?										
66.8.2																									
66.8.3		1																							
66.8.4																									
66.9.3																									
66.9.4																									
68.1	1	1							1																
29.1																	1	1							
60.3															?		?					?			
60.4													1	1	1										
62.3																									
62.4														?		1									
63.2					?																				
63.3	?																				1				
63.4																					1	1			
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66.3.3																									
66.3.4			?																						
66.4.1																									
66.4.2																									
66.4.3																									
66.4.4																									

Annex 11. Continuation. Bandmatching ananlysis with the software GELCOMPARE for all the samples with the primer OPB18.
1: confirmed band. ?: uncertain band.

Station/rep	GEL5:69.86bp	GEL6:890.96bp	GEL6:131.55bp	GEL2:349.22bp	GEL6:702.69bp	GEL6:574.79bp	GEL6:614.28bp	GEL6:661.80bp	GEL6:400.97bp	GEL6:287.76bp	GEL6:802.61bp	GEL3:227.36bp	GEL3:775.08bp	GEL3:486.39bp	GEL3:343.90bp	GEL3:250.69bp	GEL3:264.57bp	GEL3:107.96bp	GEL3:703.09bp	GEL3:695.06bp	GEL3:430.94bp	GEL3:338.44bp	GEL1:1267bp	GEL2:265.12bp	GEL1:1385bp
66.5.1	?																1		1	1					
66.5.2																	?	1							
66.5.3																									
66.5.4					?						?														
66.6.1																									
66.6.2																									
66.6.3																									
66.6.4																							1	1	
66.8.1							1	1	1	1															
66.8.2					1	1	1			1															
66.8.3						1	?			1														?	
66.8.4				?																			1	1	1
66.9.3																							1	1	
66.9.4												1													
68.1		?																							

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