Laboratory Culture Experiments on Benthic Foraminifera

Thesis submitted to the

Goa University

For the award of degree of

DOCTOR OF PHILOSOPHY
IN
MARINE SCIENCE

by

Linshy V.N.
Micropalaeontology Laboratory, National Institute of Oceanography
Dona Paula 403 004 Goa India

2010

Dedicated to

Lord Almighty . . .

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Declaration

*As required under the university ordinance OB.9.9 (ii), I hereby state that the present thesis entitled "**Laboratory experiments on benthic foraminifera**" is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned.*

Literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

-Linshy V.N.

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(वैज्ञानिक एवं औद्योगिक अनुसंधान परिषद)
national institute of oceanography
(Council of Scientific & Industrial Research)

Dr. R. Nigam, Ph.D., D.Sc.
Scientist 'G'
Project leader, Paleoclimate

Date: 28th April 2010

Certificate

*As required under the university ordinance OB.9.9. (vi), I certify that the thesis entitled "**Laboratory experiments on benthic foraminifera**", submitted by Ms. Linshy V.N. for the award of the degree of Doctor of Philosophy in Marine Science is based on original studies carried out by her under my supervision. The thesis or any part of thereof has not been previously submitted for any other degree or diploma in any university or institution.*

(R. Nigam)

Research Guide

All the corrections suggested by the Examiners have been incorporated in the Thesis.

14.10.2010

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दोना पावला गोवा 403 004 भारत
DONA PAULA, GOA- 403 004, India
☎ : 91-(0)832-2450 450
fax : 91-(0)832-2450 602/03
e-mail : ocean@nio.org
URL : **http://www.nio.org**
Regional Centres
Mumbai, Kochi, Visakhapatnam

Acknowledgement

First and foremost I would like to thank Dr. R. Nigam, my supervisor/mentor/guide for giving me a beautiful dream to pursue with a passion-the foraminiferal research and making me able to stand at this point of my career. A great teacher, as he always used to be, his encouragement, guidance and support has been a great blessing throughout this period.

I take this opportunity to express my gratitude to the former and present directors of National Institute of Oceanography, Dr. E. Desa, and Dr. S. Shetye, for facilities and continuous encouragement.

Special thanks are extended to Prof. G.N. Nayak, my co- guide, former Dean and Head, Department of Marine Sciences for his support and encouragement. Thanks are also due to Dr. A.L.Paropkari- Vice Chancellor's nominee for his constructive comments and suggestions.

I express my gratitude to Prof H.B. Menon, Head, Deptt. of Marine Sciences, and Prof. P.V. Desai, Dean, Faculty of Science for all their help and support.

I am grateful to Dr. R. Sharma (former Head) with Dr. Banakar, Head, HRDG, Mr. KrishnaKumar, Mrs. Mashik and Mr. Ghanti from the Human Resource Development Group for helping us with all administrative works. I thank Dr. M.D. Rajagopal and Dr. R.G. Prabhu Desai, former and present Head, PMN for their timely help during the hour of the need. Thanks are also due to Mrs. Soja Simon and Mr. Pathak for their valuable help at various point of time. Special thanks to Documentation officer (NIO) Dr. M. Tapaswi and his team for sincere efforts in procuring necessary literature throughout my study. I thank The DTP group, especially Mr. Shyam, Mr. Uchil for their creative help and Mr. Arun Mahale for helping with the printing of posters for various conferences. Special thanks to Mr. T.P. Simon for all the timely support extended at various stages.

I thank Dr. Leon Moodley from Netherlands Institute of Ecology (NIOO) for allowing me to participate in Indo- Dutch INDO- DUTCH OMBEF program, during which I learned *In situ* culture techniques for benthic foraminifera.

At this point of time I would like to thank all my teachers of Geology Department, Pune University, and also Scientists from Agarkar research institute for initiating research interest in me.

My heartfelt gratitude to Dr. Rajani Panchang Dhumal, who introduced me to Dr. Nigam and walked me to the wonderful world of Oceanographic research. I will remain indebted to her all through my life for all the guidance, encouragement, love and support she gave me.

Words are not enough to express my thanks to Dr. Sanjay Singh Rana for being such a wonderful friend and support all these years.

My whole hearted gratitude to Dr. R. Saraswat, for helping me with his valuable suggestions at various stages of my research.

I like to thank all my super seniors from micropaleontology laboratory, Dr. N. Khare; Dr. Henriques, Dr. Mayernkar, Dr. Chaturvedi, Dr. Mazumder and Dr. Govil for their encouragement and support from time to time.

Thanks are due for all my labmates for these last 5 years, Sujata R.K., Shanmukha D.H., Aida Fernandes, Swati Bhonsle Phadte, Lea Barreto, Yatin Fadte, Akshay Parab, Chandrashekhar, Durvesh Pednekar, Sheetal Godad, Rubina Pednekar for making my life easy and cheerful throughout. Special thanks to Sujata, Shanmukha, Rajeev, Pawan and Sanjay for the help extended in during field works; Swati for her always ready to help attitude and support; Aida for the timely help; Yetin, Akshay and Durvesh are due for very special thanks for each and every day spent with me and my little foraminifers. All my seniors and contemporaries will be remembered in life for making micropaleontology laboratory to 'Micropal family'.

My sincere thanks to Mr. Dinesh Naik for the timely help during the printing of this theisis.

A special thanks to all the summer and dissertation students- Vinay Padte, Kavita Mallya, Mrinal sawant, Pritha Chakravarty, Manish Sharma, Jasmeet Kaur who worked with and helped me in many ways during their stay.

I would like to thank all my friends in NIO and outside for their support and encouragement. This is not possible to fill out all the names here in this small page so just imagine it here. Thanks you for making life as it is.

The whole exercise of acknowledgement would be incomplete if I don't mention a special category – various funding agencies with the help of which I could convert my dreams to reality without any difficulty. Chronologically starting with Science and Society Division, Department of Science and Technology for awarding me Women Scientists Fellowship for 3 years; Council of Scientific and Industrial Research for awarding the Senior Research fellowship; University Grants Commission and Deutcher Academischer Austausch Dienst, Bonn, Germany for UGC-DAAD short term fellowship.

Few people from abroad owe special mention here- Prof. Michal Kucera, Head, Micropaleontology Group, Tubingen University Germany for giving placement and all the support for successfully availing DAAD fellowship; Dr. Petra Heinz, My Mentor in Germany for her guidance and support and all the valuable insights to foraminiferal research and special thanks for the permission to incorporate the collaborative work on stress proteins in to this thesis; Dr, Hartmut Schultz for his help and friendly time; Dr. Christoph Hemleben and Prof. Vera Hemleben for all the encouragement; Dr. Heinz Schuler and the members of Animal Physiology Group for the valuable collaboration and support for the Protein studies; Bremen University and Museum für Naturkunde Leibniz-Institut für Evolutions- und Biodiversitätsforschung an der Humboldt-Universität zu Berlin for analytical support. Mr. Romen Marten and Ms. Anne Katrin are

specially thanked for the working hours shared for the protein studies and the sampling tour to Angers France respectively. Million thanks to Ute Treppke for being such a nice company all throughout my stay.

Special thanks to Dr. Emmanuel Geslin, University of Angers, France for arranging and accompanying to field trip for collecting samples for various laboratory studies on foraminifera during my DAAD fellowship.

Dr. Zeng Shui, Department of Oceanology, China is acknowledged for helping with the species level identification of *Strebloides advena*

And to wind up with the most precious piece of acknowledgement to the most wonderful lot of people in my life- My Achan, Amma, Chettan, Teacheramma, Achu all others from the family for their motivation, support and valuable prayers. Finally to my backbone Ajesh for providing me everything with a lot of sacrifices at his personal front and made all my efforts fruitful through his tremendous support, energy and encouragement to make this dream a reality.

Thanks be to lord almighty who's never ending and unfailing love keeps me going in life...

Linshy

Foreword to thesis

Foraminifera are considered to be one of the best indicators for ecologic studies of past and present day marine environments. They are highly sensitive to the changes in the physico-chemical parameters in their ambient environment (natural as well as anthropogenic). The response to such changes may vary from variations in abundance, diversity, growth, morphology and chemistry of the hard parts. Foraminifera incorporate signatures of these changes and preserve them in their hard part called test. They are present in all marine environments and have a great diversity and abundance. The fossilization potential of the tests render foraminifera a positive edge over other micro-organisms and make them the ideal bio-indicators for short- long term changes of the marine environments, from global to local scales.

Foraminiferal research is often debated to be lying in the boundary between geology and biology. It is very much known that in order to interpret the microfossils correctly, we need to understand the biology and ecology of their living counter parts. And it is very much apt to point that this is the principle, on which the geological applications of foraminifera based on and it is commonly noted by the geologists as *present is the key to the past* (Principle of Uniformitarianism). In recent years, the number of studies on foraminiferal ecology has increased and lots of advanced techniques are attempted.

Experimental studies on foraminifera are a fast evolving and an important branch of foraminiferal research, which provides experimental proof to the field based results and thereby increasing their credibility. In most of the field studies, the foraminiferal characteristics are attributed to the site specific (the type and extent of stresses vary at different sites) field evidences; but such studies are yet to give a complete account of the specific responses of foraminifera to specific stresses, be it natural or anthropogenic. In the laboratory, physico-chemical parameters are altered in such a way that the response of foraminifera to specific parameters can be studied in isolation as well as in desired combinations also. This flexibility of the experimental studies, along with the high precision results (qualitative as well as the quantitative), makes it an indispensable part of modern foraminiferal research and help revolutionizing/updating our understanding of foraminiferal ecology for the application of the same in various paleoclimatic and pollution studies.

This thesis deals with the fundamental aspects and establishment of laboratory culture studies on few selected species of benthic foraminifera, learning their growth, reproduction and lifespan in the laboratory, their response to various stress conditions including heavy metals, oxygen etc. Additionally production of stress proteins in benthic foraminifera, and response of some foraminiferal species to ^{13}C labeled algal feed is also studied as a part of collaborative work with a German laboratory.

Comprising of ten chapters, the thesis has **Chapter 1** establishing the need of laboratory studies on benthic foraminifera in view of the rudimentary stage of the same in this part of the world. Realizing the fact that Indian scientific world is still to attempt and urging the need of experimental studies to evaluate the field based results, a laboratory with advanced facilities was set-up in National Institute of Oceanography, Goa and a dedicative workgroup is continuously attempting to study various aspects of foraminiferal response (emphasizing hard part alterations) to known parameters. The results discussed in this thesis are a part of the research conducted in the Foraminiferal culture laboratory, National Institute of Oceanography, Goa, acclaimed to be one of its kinds in India.

Chapter 2 is a detailed review of the studies done worldwide on the laboratory studies on benthic foraminifera. As much as 164 papers have been reviewed and the facts emerged is as follows-

- Most of the studies were done by biologists who were interested in studying the soft part of foraminifera.
- Studies dealing with the hard part of foraminifera were comparatively less and the proper understanding of the parameters affecting the morphology of the benthic foraminifera is still lacking.
- Exact mechanism leading to the test deformities is yet to be explored
- Characterization of foraminiferal response to pollutants so as to effectively use it as a proxy for marine pollution studies.

Taking these facts in to account, I decided to work on laboratory culture experiments on benthic foraminifera for the doctoral thesis and the following objectives were set-

- To establish culture program and to observe life span and mode of reproduction of selective foraminiferal species
- To observe the response of benthic foraminifera to specific pollutants
- To characterize type of abnormalities (if any) in benthic foraminifera as a result of different pollutants

In **Chapter 3**, a detailed sketch of general methodology involved in the laboratory culturing of coastal benthic foraminifera is given. The experimental set-up of different experiments is discussed in the respective chapters for better understanding of the procedures. This chapter mainly deals with the field collection and preparation of samples for live culturing, separation of live specimens, maintenance in the laboratory etc. Working photographs are included to understand the field and field procedures better.

Discussion on the experimental studies carried out on benthic foraminifera starts with **Chapter 4.** In this chapter the growth, reproduction and lifespan of 2 benthic foraminiferal species viz. *Strebloides advena* and *Rosalina leei* are discussed. Observations from consecutive generations revealed the length of the reproductive span of these species as 17-19days for *Strebloides advena* and 105-109 days for *Rosalina leei* and previous studies confirm that these are the first report of lifespan of *Strebloides advena.*

In **Chapter 5**, foraminiferal responses to varying levels of oxygen is discussed with the help of an experiment conducted onboard ORV Sagarkanya, followed by subsequent observations in container lab brought from NIOO, The Netherland. The main findings indicate that genera like *Fursenkoina* and *Nonion* showed more adaptive values to changing oxygen conditions and migrated to more oxygenated surface layers, whereas the *Rotalids* and *Bolivinids* died out showing poor adaptation to such stress conditions. The results thus obtained will be useful to explain the occurrence/abundance of certain foraminiferal species under oxygen minima conditions in the past.

Chapter 6 discusses foraminiferal response to anthropogenic stress by citing examples of two common heavy metal pollutants along our coast line viz. Mercury (Hg) and Cadmium (Cd). Experiments conducted on benthic foraminiferal species *Rosalina leei* to study their response to different levels of heavy metal Mercury is included in the beginning of the chapter followed by the experiments on *Pararotalia nipponica* with heavy metal cadmium. The results from the experiments confirm the effective application of foraminiferal proxies for marine pollution studies.

After learning the morphological responses of foraminifera to different stress conditions, attempts were made to understand the production of stress proteins in

benthic foraminifera **(Chapter 7)**. The total protein content per individual was also determined for the benthic foraminiferal species under study.

The last experiment **(Chapter 8)** included in the thesis deals with the response of benthic foraminiferal response to ^{13}C labeled algal feed. Experiments were conducted on benthic foraminiferal species *Ammonia tepida* and the results show the preference of this species to fresh organic matter and its pattern of food uptake with time.

Thesis is concluded with **Chapter 9** which summarizes the major findings of the present work and indicates the potentials of future research in this direction. References cited in the thesis are included as a separate list after this chapter. The list of publications and the certificates of merit are enclosed as Annexures.

CHAPTER 1

INTRODUCTION

Chapter 1

Introduction

"Our basic concern is with establishing species successfully in the laboratory, so that they grow to maturity, reproduce at a reasonably normal rate, and thus provide populations that constitute a permanent and dependable source for further study"

-Zach M. Arnold

Perspective is one of many illusionary aspects of life. Micropaleontologists, paleoecologists, physical, geological and biological oceanographers, protozoologists and marine microbiologists very often look at foraminifera from quite different vantage points (Lee, 1974). Being a Micropalaeontologist, my interest in foraminifera has been very distinct in a way. Having known the vivid applications of fossil foraminifera, I was drawn to this field, but ultimately ended up culturing living benthic foraminifera in laboratory. Interestingly I landed up and happened to work in a laboratory which happened to be India's only laboratory to deal with laboratory studies on foraminifera.

1.1. Foraminifera: A short introduction

The foraminifera are enormously successful group of amoeboid protists. The name 'foraminiferans', from the Latin (meaning aperture bearers) was first coined in 19th century by the great French naturalist Alcide Dessalines d'Orbigny. Branching very deeply within the eukaryotic evolutionary tree, they first appeared in the Cambrian and over the course of the phanerozoic, invaded most marginal to fully marine environments and diversified to exploit a wide variety of modes in life (Goldstein, 1999). Since then, they have radiated and evolved to make up approximately 40,000 species in the rock record. Based on the habitat they live, foraminifers are generally grouped as planktonics and benthics in which the former are water column floaters and are abundant in the deeper waters where as the latter are bottom dwellers and are most abundant and diverse in coastal waters.

The principal characteristics of foraminifera are (1) threadlike anastomosing pseudopodia bearing granules (granuloreticulopodia) that reveal constant bidirectional streaming of the cytoplasm; (2) the life history characterized by an alteration of sexual and asexual generations with meiosis associated with the asexual reproduction - a feature unique in heterotrophic eukaryotes; and (3) the presence of a test (shell). The test can be composed of biogenic calcium carbonate (calcareous), cemented foreign particles such as quartz

grains (agglutinated), or an organic theca composed of polysaccharides. Few naked foraminifers have been found as well, but they are found in fresh water where as all others are marine.

The biology and ecology of modern foraminifera is comparatively poorely known, partly because the group has historically received more attention from geologists than biologists and partly because of the difficulties experienced in reproducing the living conditions in the laboratory culture experiments.

When applications are considered, foraminifera possess very different and distinct qualities compared to other micro-organisms in general. They are present in all marine environments (ubiquitous) and have a great diversity and abundance. Usually a few millimeters of sediment contain several hundreds of individuals and several species. They are highly sensitive to the changes in the physico-chemical parameters (natural as well as anthropogenic) in their ambient environment. The response to such changes may vary from variations in abundance, diversity, growth, morphology and chemistry of the hard parts. Foraminifers incorporate signatures of these changes and preserve them in their hard part usually known as test. When they reproduce or die, their tests become part of the sediment and subsequently the fossil record. The fossilization potential of the tests facilitates studies even long after the death of these organisms and render them a positive edge over other micro-organisms and make them the ideal bio-indicators for short- long term changes of the marine environments, from global to local scales in both space and time (For Indian region refer reviews by Nigam & Khare, 1995; Kathal, 1998; Bhalla *et al.*, 2007; Khare *et al.*, 2007; Saraswati, 2007; Sharma & Srinivasan, 2007; Singh, 2007; Sinha, 2007)

1.2. Experimental studies on benthic foraminifera: A brief outline

Benthic foraminifers have been studied extensively worldwide for decades, and in most of the field studies, the foraminiferal characteristics are attributed to the site specific (the type and extent of stresses vary at different sites) field evidences; but such studies are yet to give a complete account of the specific responses of foraminifera to specific stresses. The underlying causes affecting their distribution and morphology may be so complex that it is difficult to assign the observed patterns to a particular parameter, as to whether it is natural (salinity, temperature etc.) or anthropogenic (pollution). For a species or an assemblage to be useful as a proxy, its abundance must show a strong correlation with the chosen factor. Hence we need to learn more about the factors (ecology) that cause variations in their abundance as well as morphology. Therefore it is essential to evaluate the accuracy of

existing techniques and develop new techniques to refine benthic foraminifera as an efficient proxy for various applications.

Experimental studies on foraminifera are a fast evolving and an important branch of foraminiferal research, which provides experimental proof to the field based results and thereby increasing their credibility. In the laboratory, physico-chemical parameters are altered in such a way that the response of foraminifera to specific parameters can be studied in isolation as well as in desired combinations also. Laboratory studies allow-

- Continuous close monitoring of vital activities
- Record of response to specific parameters
- Understanding of favorable and non-favorable factors
- Delineation of natural and anthropogenic factors
- Evaluation of existing techniques & generation of new techniques

This flexibility of the experimental studies, along with the high precision results (qualitative as well as the quantitative), makes it an indispensable part of modern foraminiferal research and help revolutionizing/updating our understanding of foraminiferal ecology and morphology for the application of the same in various paleoclimatic and pollution studies.

The biology of foraminifera has not been examined as intensively as that of other marine protists. There are several inherent properties that render foraminifera as a group more difficult to handle experimentally than numerous other popular laboratory protozoa, principal among which are-

- Their relatively slow rate of reproduction and comparatively long life-span, often with the additional complication of alternation of generations.
- Their comparatively subtle vegetative activities and the consequent difficulty of accurately assessing their general condition.
- The remoteness of many labs from the sea and the easy access to living organisms.
- Long standing misconceptions about the handling/ maintenance of marine cultures.
- The general lack of information about the optimal conditions required for the successful maintenance of laboratory cultures, particularly the nutritional requirements.

Probably for the same reasons, the earlier studies on foraminifera were limited. As geologists began to recognize the value of foraminifera in hydrocarbon exploration and paleoecological interpretations, they stimulated interest in ecological studies of

foraminifera but found so few biologists willing or prepared to undertake them that they were often forced to do so themselves (Arnold, 1974).

The species a paleontologist would select for biological study are not necessarily those a biologist would, because the two specialists have different interests and needs. The former would naturally prefer to learn more about the biology of species important to the interpretation of the fossil record. This generally means species having preservable hard parts, i.e. calcareous or agglutinated tests. Ideally the species should be the same as its fossil counterpart or in the case of one now extinct, its closest living relative. Moreover paleontologists require species whose individual representatives are large enough for effective statistical correlations (Arnold, 1974)

The increasing paleontological need for the fundamental biological processes of foraminifera for a better interpretation of the field based foraminiferal findings stimulated the laboratory studies during the past few decades. Some of the previous reports and our laboratory experience reveal that some of the common inter-tidal foraminifera are not only easier to procure, they are easier to maintain in the laboratory with relatively simple facilities compared to the forms from the deeper water particularly planktonics which require sophisticated laboratory set-ups.

Considering the proximity to the field for effective sample collection and the laboratory facilities created, right now I have restricted to the study of coastal water benthic fauna. It is also very much essential to admit here that there are several species which were brought to laboratory in living conditions, but could not be retained living in the laboratory for several reasons and there are some other species which continued to live in the laboratory conditions but did not reproduce at all. Still considering the limited information available on the laboratory behavior of foraminifera, every bit of information contributes to the record.

1.3. Experimental studies on benthic foraminifera: Indian perspective

Though popular and much advanced in other countries (Arnold, 1954a; Bradshaw, 1961; Sliter 1970; Kitazato, 1988; Moodley *et al.*, 1998a; Heinz *et al.*, 2002) laboratory studies of foraminifera are yet to attain proper attention in this part of the world. Review of literature (Linshy *et al.*, 2007: included as 3rd chapter) and also special mention in some of the publications support our understanding that 'Foraminiferal Culture Laboratory' of National Institute Oceanography is the only place in India attempting this particular aspect of foraminiferal research (Nigam *et al.*, 1996a, 1996b; Saraswat *et al.*, 2004). Well

established in the field of micropaleontology for more than 3 decades, the micropaleontology workgroup has considerable number of novel results to their credit. Over the years of the research, some of their innovative field hypothesis (like *the application of Mean Proloculus Size* (Nigam & Rao, 1987); *Dimorphic Ratio of benthic foraminifera for paleoclimatic studies* (Nigam, 1986); *morphological abnormalities in foraminiferal test for marine pollution studies* (Setty & Nigam, 1984) germinated the idea of refinement of field results through laboratory (Nigam, 2005; Nigam *et al.* 2006a). Motivated/Inspired from the field findings, a project was written on the laboratory culture studies on benthic foraminifera in collaboration with Woods Hole Oceanographic Institution, U.S.A. under BOYSCAST fellowship from DST in the year 1990 which marked the beginning of Benthic Foraminiferal Culture Studies in India and also the set-up of 'Foraminiferal Culture Laboratory' in National Institute of Oceanography, Goa.

Initially, experimental studies were attempted as a part of the fossil studies and no additional infrastructure was available to the work group. Gradually with the successful culturing of few coastal benthic foraminiferal species and with the publication of initial results, more and more infrastructure was developed in the laboratory with financial aid from various funding agencies. In the year 2005, two fulltime projects on laboratory culture of foraminifera was awarded to the laboratory by DST under two different schemes (SERC to work on the laboratory culture of foraminifera to develop paleoclimate proxies; SSD to develop foraminiferal proxies for effective marine pollution monitoring), the funding of which helped the lab to attain the present status with all the sophisticated and modern facilities to attempt more refined research on various experimental aspects on benthic foraminifera. A dedicated workgroup under the guidance and training from Dr. Nigam is continuously attempting various aspects of laboratory experiments in this laboratory and publications from this workgroup over the years are tabulated in chapter 2 (Table 2.2).

This thesis is a part of the research carried on in the Foraminiferal culture Laboratory, National Institute of Oceanography, Goa, India with the timely award of fellowships from Department of Science and technology (in the form of Women Scientist Award by the Science and Society Division from March 2005-March 2008) and Council of Scientific and Industrial Research (in the form of Senior Research Fellowship from April 2008 till date). Part of the thesis was done in the Micropaleontology laboratory, Eberhard Karls University, Tubingen, Germany under short term UGC-DAAD fellowship (from May 2008-November 2008).

1.4. Emphasis of the present study

Considering the rudimentary state of laboratory studies of benthic foraminifera in India, much emphasis is given to the establishment of the same by various ways and means possible ranging from the development of sophisticated infrastructure in the laboratory to presentation/discussion of results and research ideas in various national and international conferences. As mentioned right in the beginning of the thesis, the basic concern was with establishing as much as benthic foraminiferal cultures successfully in the laboratory, so that I can understand their basic functions like the growth phases, maturity, mode of reproduction at a reasonably normal rate, so as to provide culture populations that constitute a permanent and dependable source for further study for the future researchers who are interested to continue from where I stop. When I write 'where I stop' it may raise so many eyebrows, but for me this is the very fact in research, we always start searching (RE-search) from where somebody stops. In view of the fact that this will be the first research thesis on the laboratory studies on benthic foraminifera from India, a detailed review of the studies done worldwide on the laboratory studies on benthic foraminifera was done prior to setting the objectives. The knowledge of some of the important physiological processes of foraminifera (like their lifespan, mode of reproduction, response to specific parameters etc. in order to efficiently interpret the field results was also accounted for. Efforts were made to evaluate /support some of the field based findings based on benthic foraminifera as proxies for various climatic and pollution studies through the laboratory studies.

CHAPTER 2

REVIEW OF LITERATURE & OBJECTIVES OF THE PRESENT STUDY

Chapter 2

Review of literature & objectives of the present study

"Over hundreds of millions of years these tiny creatures have swarmed the ocean bestowing to the planet their exquisite dwellings, a smidgen of which was once fashioned into the Egyptian Pyramids"

- Anonymous

2.1. Introduction

The laboratory culture studies, carried out on benthic foraminifera, with the aim to refine paleoceanographic/paleoclimatic or environmental application of benthic foraminifera, have been reviewed. The review includes studies, which refined the understanding of factors that bring out changes in benthic foraminiferal abundance, morphology, isotopic and chemical composition (of the test). Additionally, studies dealing with taxonomic aspects of benthic foraminifera have also been discussed, since such studies have significantly improved application of benthic foraminifera for stratigraphic correlation. The International and National reports dealing with the experimental studies on benthic foraminifera are discussed separately.

2.2. State of art: International

In order to get a clear idea of the emphasis given to different aspects of experimental research, the international review is divided in to the following sections-

- Studies carried out to understand the factors affecting abundance and morphology
- Studies that helped understand the factors affecting chemical composition
- Studies that refined evolutionary position of foraminifera
- Studies that helped to identify cryptic species
- Miscellaneous studies that refined application of benthic foraminifera for past climatic/oceanographic reconstruction.

The discussion of the state of art would be incomplete without the details of the previous reviews published by earlier workers on related topics; hence the chapter begins with the current status of reviews done till date.

2.2.1. Previous Reviews

Earlier Lister (1895) compiled the studies covering the biological aspects of foraminifera but the results were mainly based on the field observations. Later on, efforts were made to provide a comprehensive review of the factors affecting the abundance and growth of species, as well as its response to various ecological parameters based on the samples collected from the field (Myres, 1943b). Murray (1973) compiled the biological aspects of foraminifera and their potential application for paleoecological studies, but the findings included observations made both in the field and laboratory culture. A comprehensive review of ecological parameters affecting the benthic foraminiferal morphology was provided by Boltovskoy *et al.* (1991). But, here also, most of the studies included were based on the field observations. Subsequently, the paleoceanographic significance of studies carried out to understand the biological aspects of benthic foraminifera was compiled and discussed by Gooday (1994). In this study, results from laboratory culture of benthic foraminifera were included to some extent, much emphasis was given to the influence of food and oxygen conditions on the benthic foraminiferal communities. Thus, a complete review of the studies carried out to understand the specific effect of a single, or a combination of few ecological parameters on benthic foraminifera under laboratory culture, is not available.

As the culturing of foraminifera increased with advancements made in the techniques to maintain benthic foraminifera under laboratory conditions, a brief historical account of developments in the benthic foraminiferal culturing techniques is given in the next section.

2.2.2. Laboratory culturing techniques through ages

Though laboratory maintenance of benthic foraminifera started from the first half of the 19th century and number of studies were published during the later half of the 19th century, it was not until the mid of 20th century that ecological experiments were conducted on benthic foraminifera maintained in laboratory culture. Myers (1935a, 1937) discussed various methods and precautions to be taken during collection and maintenance of benthic foraminifera from the littoral zone in the laboratory. Arnold (1954a) and Slater (1955) provided a comprehensive review of the various methods to culture benthic foraminifera and proposed a simple system for laboratory maintenance of benthic foraminifera. Subsequently, Lee *et al.* (1961) and Arnold (1966, 1974)

provided a detailed description of the methods of collection and maintenance of living foraminifera in the laboratory, as a part of their research on growth and physiology of foraminifera in the laboratory. An updated account of the developments made in the laboratory culturing methods for benthic foraminifera was given by Anderson *et al.* (1991). Recently, Hintz *et al.* (2004) described a new setup to maintain benthic foraminifera under controlled physico-chemical conditions under laboratory conditions. It will help to carry out studies to understand the specific physico-chemical and biological factors affecting chemical composition of the foraminiferal tests, one of the most frequently used foraminiferal characteristic.

Most of these studies discussed the methods for maintaining shallow water benthic foraminifera under laboratory conditions. Though limited success was reported in maintaining deep-water benthic foraminifera under normal laboratory conditions (Weinberg, 1990), successful maintenance of deep-sea foraminifera under high pressure and low temperature conditions paved the way for laboratory culturing of deep-sea benthic foraminifera (Turley *et al.*, 1993).

However, the laboratory culture response should be taken with care as significant differences have been observed in the species behaviour under similar conditions (Röttger & Berger, 1972). Schnitker (1967) observed that under laboratory culture, specimens of *Triloculina linneiana* attained sexual maturity at one-eighth the size of the parent specimens recovered from the field and were morphologically different from the parents.

2.2.2.1. Studies on the factors affecting abundance and morphology

Temporal changes in the benthic foraminiferal abundance and morphology are the most commonly used foraminiferal characteristics (Boltovskoy & Wright, 1976; Murray, 1991; Sen Gupta, 1991) for assessment of present and past environments. However, despite an apparent link between abundance, morphology and ecological parameters, how much change in a particular parameter will bring a specific change in the faunal characteristics, is still not clear (Gooday, 2003). The morphological changes from the field have almost always been assigned to the circumstantial ecological stress including both natural extreme physico-chemical conditions and induced pollutants (Boltovskoy *et al.*, 1991), with a lack of understanding of precise cause of specific morphological changes. Contrasting results from the field have further complicated the issue (Geslin *et al.*, 2002). Therefore numerous culture studies

have been carried out to understand the factors that bring out a known change in the benthic foraminiferal abundance and morphology.

A. Effect of type and amount of food

Significant seasonal differences in the foraminiferal population and assemblage (Gooday, 2003; Gooday & Lambshead, 1989), lead to the belief that changes in the type and amount of food may be one of the factors responsible for the changes in the foraminiferal population. Therefore, it was imperative to understand the effect of changes in the phytodetritus on the benthic foraminiferal population.

Based on foraminiferal culture studies it was observed that long-term changes in the foraminiferal diversity and abundance were brought by the amount of food and the response was species specific. Within the same genus, certain species preferred live food whereas others were found to thrive well when fed on dead cells (Bradshaw, 1955). Ernst *et al.* (2005) observed that the foraminiferal response to the amount of dissolved oxygen and food was species specific wherein certain species (*Stainforthia fusiformis*, *Nouria polymorphinoides*, *Hopkinsina pacifica*, *Nonionella turgida*) responded quickly to the changes in the dissolved oxygen while the others (*Caronia silvestrii*, *Epistominella vitrea*, *Acostata mariae*) responded only to the changes in the amount of food. The food uptake rate, as well as the ratio of accumulated to digested quantity of food, changed from species to species and also for different sized individuals of the same species (Moodley *et al.*, 2000; Moodley *et al.*, 2002; Langezaal *et al.*, 2005). The study can help to understand the changes in the species assemblages and the abundance of certain species under changing oxygen and food conditions in the field. Extreme scarcity of food leads to the cessation of growth and reproduction in certain species while the unfed specimens of certain other species grow equally well or better than the specimens that were supplied with food at regular intervals (Bradshaw, 1961; Lee *et al.*, 1991). However, the scarcity of food lead to longer survival rate as the reproduction was delayed (Hemleben & Kitazato, 1995). The study can help understand the reduced abundance of few species under low productivity conditions. The response to additional essential nutrients, nitrate or phosphate, was not only species specific but also was different under different light and food conditions. While *Amphistegina lobifera* does not respond to changes in nitrate and phosphate, *Marginopora kudakajimensis* showed maximum growth under illuminated, nutrient enriched and well fed conditions (Lee *et al.*, 1991). The type of

food also affected the growth rate as well as the survival of benthic foraminifera (Lee *et al., 1961;* Lee *et al.*, 1991). Lee and Muller (1973) noted that *Allogromia laticollaris*, *Rosalina leei* and *Spiroloculina hyalina* were selective feeders preferring only few algal species. Furthermore, these species can adjust well with the changes in the microbial community structure. The essential presence of bacteria for favorable growth was reported by Lee *et al.* (1991).

The response to phytodetritus has also been found to vary as per the depth at which the species are found in the ocean, thus reflecting the depth zonation of benthic foraminiferal species. Witte *et al.* (2003) noticed that the response of abyssal foraminifera to the phytodetritus was different than that of the continental slope species and foraminifera showed a retarded response as compared to the macrofauna. Nomaki *et al.* (2005a) studied the carbon assimilation rate of benthic foraminiferal species under *in situ* experiments and noted that shallow infaunal species (*Uvigerina akitaensis, Bulimina aculeata*) assimilated more carbon as compared to the intermediate (*Textularia kattegatensis*) and deep infaunal species (*Chilostomella ovoidea*). Here also, the response varied within the same species and also species to species subject to different types of food during different seasons. It thus explains the changing abundance and diversity during different seasons and in different geographical regions. The arrival of food has increased impact on the shallow infaunal species as compared to the deep infaunal species, while few species do not respond at all (Nomaki *et al.*, 2005b).

Linke (1992) identified two survival strategies in benthic foraminifera based on the adenosine-tri-phosphate (ATP, a measure of feeding activity) content and metabolic rates, namely, the one that maintained uniform rate throughout the year and those that showed seasonally varying ATP turnover rate. The latter group of species will increase in abundance and respond well to the increased phytodetritus input. Different feeding preferences and mechanisms in benthic foraminifera were identified by Nomaki *et al.* (2006), who reported that benthic foraminifera prefer atleast three types of food (1) fresh phytodetritus, selectively (phytophagous species, namely *Uvigerina akitaensis*, *Bolivina spissa*, *Bolivina pacifica)*; (2) fresh phytodetritus, selectively but sedimentary organic matter as well, when phytodetritus is absent (seasonal-phytophagous species, namely *Bulimina aculeata*, *Textularia kattegatensis*, *Globobulimina affinis*); and (3) sedimentary organic matter at random (deposit feeders, namely *Cyclammina cancellata*, *Chilostomella ovoidea*). The response of

benthic foraminifera to phytodetritus under controlled laboratory culture studies has definitely helped to understand the changing abundance of different species in the field.

B. Effect of oxygen concentration

The amount of phytodetrital material reaching to the seafloor is one of the factors that regulate the concentration of dissolved oxygen in the bottom water and pore water, the regions inhabited by benthic foraminifera. Therefore, food and oxygen have been suggested as two important parameters that define the microhabitat of benthic foraminifera (Jorissen, 1999). The relative influence of food and oxygen concentration on benthic foraminiferal community is debated.

Moodley *et al.* (1998b) noted that several foraminiferal species can survive under anoxic conditions and soft-shelled foraminifera were comparatively less tolerant to anoxia. Heinz *et al.* (2001) noted increased population density under high oxygen and food condition and that the migration of species within the sediments was controlled by the availability of oxygen. Geslin *et al.* (2004) also noted that the oxygen concentration defined the epifaunal or infaunal habitat of benthic foraminifera and further that though few species were strictly oxic, certain others could tolerate both oxic and anoxic conditions, probably explaining the changing abundance of benthic foraminifera under changing oxygen conditions in the field. Changes in oxygen concentration also result in considerable change in the subsurface distribution of benthic foraminifera as well as the migrational capability of benthic foraminifera (Gross, 2000). However, Bernhard (1993) did not report any statistically significant change in the foraminiferal survival rate or the adenosine-tri-phosphate content of the foraminifera subjected to different oxygen levels. But in a subsequent study Bernhard & Alve (1996) reported significantly reduced ATP concentrations in species subjected to anoxic conditions.

C. Effect of light and symbionts

Symbionts are one of the essential aspects of certain benthic foraminifera and their possible influence on foraminifera has been discovered long ago (Cushman, 1930). The photosynthetic activity of symbionts depends on the light conditions, which vary with the seasons and depth, in the marine habitats. Therefore, food intake of certain algal symbiont bearing benthic foraminiferal species is affected by the changes in the

light intensity. Thus, in laboratory culture experiments carried out to understand the effect of light intensity on the benthic foraminifera, much emphasis is given to the symbiont bearing benthic foraminiferal species.

Lee *et al.* (1979) suggested that the presence of symbionts in the foraminiferal tests was responsible for the larger size attained by certain benthic foraminifera. Differences were noted in the response of symbiont bearing benthic foraminiferal species and the ones devoid of symbionts, to food. It was reported that the food intake by certain symbiont bearing benthic foraminifera was proportional to the density of chloroplast present, whose abundance changed significantly with the changing light-dark conditions (Lopez, 1979). Kuile & Erez (1984) showed that the growth rate decreased significantly under dark conditions in symbiont bearing benthic foraminiferal species, whereas Lee *et al.* (1961) reported that the symbiont bearing species could not survive prolonged darkness. Photoinhibition also varied from species to species. While the *Amphisorus hemprichii* and *Amphistegina lobifera* were photoinhibited above 200 klx illumination, similar phenomena was observed in *Amphistegina lessonii* and *Heterostegina depressa* at lower than 10 klx light intensity (Lee *et al.*, 1980). Williams & Hallock (2004) simulated the bleaching conditions in laboratory and noted that the growth rate of *Amphistegina gibbosa* increased considerably under blue light while ultra-violet light towards the lower wavelength does not have any apparent effect on the growth rate; however, higher end UVB considerably reduced the growth.

Except the survival and growth rate, light intensity also affect the morphology of the benthic foraminiferal tests. Growth rate initially decreased and the growth stopped slowly, when *Heterostegina depressa* were subjected to very high light intensity. The reduction in light intensity resulted in reduction in growth as well as test thickness to diameter ratio of various species of *Amphistegina* (Hallock, 1981; Hallock *et al.*, 1986). Additionally the rapidly growing tests under low light conditions tend to be comparatively more fragile and weaker (Röttger & Berger, 1972). In a significant study the growth pattern of *Heterostegina depressa* changed, once it was subjected to low illumination or complete darkness. Interestingly, different specimens showed different rate and pattern of recovery from the temporary cessation of growth under low light intensity (Röttger, 1972b).

D. Effect of seawater temperature

Seawater temperature is one of the important parameters required to understand the global climatic changes. Therefore benthic foraminiferal characteristics have increasingly been refined to track past seawater changes. In view of this many laboratory culture studies have focused on understanding the response of benthic foraminifera to changing seawater temperature.

Laboratory culture experiments show that the temperature response of benthic foraminifera as well as the lethal temperature limit is species specific (Bradshaw, 1961). The optimum temperature for a species may be anywhere between the lower and higher temperature extremes under which the species are reported from the field (Arnold, 1954a). Comparatively lower seawater temperature (<10°C) resulted in lower growth rate and cessation of reproduction in few rotalid species (Bradshaw, 1957; Bradshaw, 1961). Death of specimens was the extreme effect of temperature lower than lower thermal limits, this together with the cessation of reproduction explain the reduced abundance of certain species during the colder periods (Arnold, 1954a). The growth rate was found to be significantly low in the *Heterostegina depressa* specimens subjected to low temperature. However it does not affect the size of the chambers and in turn the shape of the test (Röttger, 1972a). The growth rate increased with increasing temperature within the temperature tolerance limits but larger size was attained by specimens subjected to comparatively lower seawater temperature (Lee *et al.*, 1991; Bradshaw, 1957). The lower temperature has adverse effect on reproduction (Myers, 1935b). Effect of seawater temperature on the sexual reproduction was explored by Nigam & Caron (2000) who observed a direct relationship between pairing in *Rosalina leei* and the seawater temperature (15°C- 15.3%, 20°C- 17%, 25°C- 19.3% pairing); however no reproduction was observed in these paired specimens under laboratory culture. Change in seawater temperature also affected the migrational capability of benthic foraminifera, though the response was different for different species (Gross, 2000).

E. Effect of seawater salinity

Change in the seawater salinity is the major factor that controls the benthic foraminiferal population and species diversity in the coastal near-shore regions. The salinity changes in these regions are mainly controlled by the fresh water input from the adjacent continental regions. The fresh water input varies as per the changes in the

rainfall intensity. Thus the temporal changes in benthic foraminiferal characteristics from the shallow water regions have been used as potential proxy to understand monsoon intensity in the past (Nigam, 2005). However, differences in the living and dead population lead to the belief that post-depositional taphonomic processes may alter the benthic foraminiferal diversity and distribution in the marginal marine areas (Murray & Alve, 1999) thus altering the original signatures. As mentioned above, one important physical parameter having potential to influence the benthic foraminiferal population in the marginal marine areas is seawater salinity (De Rijk, 1995). Therefore, laboratory culture studies have also been carried out to understand the changes in benthic foraminiferal characteristics under different salinity conditions.

Usually the salinity tolerance limit of benthic foraminifera is wider than the thermal tolerance limit (Arnold, 1954a). Both higher (30.2‰) and lower (26.8‰) than optimum salinity resulted in reduced growth, providing a possible explanation for the stunted specimens reported from the field from the regions that are subjected to increased salinity (Bradshaw, 1955). Extreme salinities (<13‰ and >40‰) also lead to delay or absence of reproduction (Bradshaw, 1961; Bradshaw, 1957). However, Freudenthal *et al.* (1963) opined that the higher salinity (36-50‰) is correlated with early reproduction. Though abnormal specimens were reported at normal salinity (37‰) also, increased abundance of abnormal specimens was reported under hypersaline (50‰) conditions. However, the type of abnormality was different in both the cases; while the abnormalities in the specimens under normal saline conditions included a double test, protruded spiral side or abnormally arranged first few chambers, the hypersaline conditions lead to abnormal proloculus, abnormally oriented coiling plane of initial chambers, multiple whorls originating from proloculus and fusion of juveniles. (Stouff *et al.*, 1999a; Stouff *et al.*, 1999b). The salinity effect studies are helpful in assessing the changing monsoon intensity based on the changes in benthic foraminiferal abundance, diversity and morphology.

F. Effect of seawater pH

The seawater pH ranges from 7.5 to 8.5, well above the acidic range. The calcareous foraminiferal tests are sensitive to seawater pH and changes in seawater pH may lead to dissolution of the calcareous tests. A significant decline in the calcareous benthic foraminiferal diversity and abundance, after deposition of the tests, was attributed to differential dissolution probably as a result of lower than normal pH of the pore water

or destruction of the tests due to current activity (Murray & Alve, 1999). Thus efforts have been made to understand the role of seawater pH on the benthic foraminifera.

The benthic foraminiferal response to changes in seawater pH is also species specific like the other physical parameters, with few species being very sensitive to slight change in pH while other tolerating a wide pH range (Bradshaw, 1961). Neutral pH leads to the dissolution in the foraminiferal tests and the dissolution starts from the last chamber and slowly the complete tests start dissolving. An interesting finding is that the specimens are able to regenerate the test, if subjected to lower than normal pH for only a short duration; however the regenerated tests are abnormal (Cadre *et al.*, 2003). McEnery & Lee (1970) also reported the regenerative capability of *Rosalina leei* and *Spiroloculina hyalina.* Angell (1967), based on the laboratory culture experiments on *Rosalina floridana* concluded that all those species that secrete an additional calcite layer over the whole test, every time a new chamber is formed, can recuperate from the dissolution incurred as a result of acidic pH. However, no separate specific mechanism especially to overcome dissolution or physical injury to the test was observed. These laboratory culture studies helped to understand the probable cause of increased abundance of abnormal species from certain location at present as well as at certain level in the geologic past. However, additional efforts are required to assign specific abnormalities to specific change in salinity or any other parameter.

G. Effect of pollutants

Differences in the foraminiferal characteristics, including changes in abundance and diversity, as well as increased abundance of abnormal specimens, have been noted from polluted environments. However, most of these observations were based on the circumstantial presence of induced pollutants. Similar effect in the benthic foraminiferal diversity and abundance has also been reported from naturally ecologically stressed environments, making it difficult to apply these benthic foraminiferal characteristics to infer past environmental conditions (Boltovskoy *et al.*, 1991; Nigam *et al.*, 2006b). Thus laboratory culture studies have been carried out to understand the effect of pollutants on the benthic foraminiferal abundance, diversity and morphology.

Initial laboratory culture studies showed that introduction of pollutants in the ambient environment of the benthic foraminifera, especially shallow water forms, leads to

significant decline in the density as well as diversity. LeFurgey & St. Jean (1976) noted that the benthic foraminiferal population as well as the species diversity significantly decreased after the introduction of sewage effluents. Though clear differences have been noted in case of faunal density and morphology in the sewage affected and normal field environments (Watkins, 1961), Topping *et al.* (2006) observed no effect of sewage derived particulate organic matter on the food source or diet of *Ammonia beccarii and Haynesina germanica.* Ward *et al.* (2003) also noted that *Haynesina germanica* does not feed on sewage derived particulate organic matter. The findings could not resolve how the sewage discharge affects the foraminifera. Additionally Alve & Olsgard (1999) showed that the extreme copper concentration (>200ppm) also, does not defer the foraminifera from colonizing the polluted sediments. Even no correlation was observed in the abundance of abnormal specimens and copper contamination.

However, further laboratory culture studies showed the effect of pollutants on the reproduction in benthic foraminifera. Few species reproduce quickly, when subjected to oil polluted seawater (Ernst *et al.*, 2006) However, in another study Cadre & Debenay (2006), observed significant reduction in the growth rate as well as reproduction, in *Ammonia* (*Ammonia beccarii and Ammonia tepida*) under high copper (200µg/l) contaminated conditions. Number of juveniles also reduced considerably and more number of abnormal tests was observed. Ernst *et al.* (2006) also noted increased number of abnormal forms under polluted conditions (experimentally induced oil pollution). The studies clearly explain the field based findings by Yanko *et al.*, (1999) reporting decreased faunal density, more number of stunted and deformed benthic foraminiferal specimens from polluted environments.

However, in an interesting study, Gustafsson *et al.* (2000) noted that the foraminiferal abundance increased after the introduction of small amount (0.02 mmol/g) of Tri-n-butyltin, probably due to the adverse effect of TBT on predators. However, increased TBT concentration (2.00 mmol/g) resulted in an adverse effect on benthic foraminifera. An example of interaction between different types of pollutants and resultant effect on foraminifera was seen by Bresler & Yanko (1995). It was observed that the presence of unidentified natural organic compounds in the seawater medium decreased the toxic effect of heavy metals on the benthic foraminifera. It can probably explain the noted differences in the foraminiferal response at different locations that have similar levels of heavy metal pollution.

H. Effect of reproduction on the test morphology

Species identification in foraminifera is entirely based on the morphology. Morphological basis of species identification has lead to the recognition of individuals with slight morphological differences as separate species, while individuals with large differences have sometimes been clubbed as same species. Initial laboratory culture studies revealed a link between mode of reproduction and morphological differences between different ontogenetic stages of the individuals of the same species. Though, the differences in number of chambers, proloculus size and overall test size were noted in almost all the species maintained in laboratory throughout their life-cycle, Myers (1936) observed a potential link between the mode of reproduction and coiling direction in *Spirillina vivipara*. Later on Myers (1940) reported that megalospheric specimens preferentially coiled sinistrally while the microspheric ones coiled dextrally in *Discorbis patelliformi*. In a similar study Lee *et al.* (1963) observed that the coiling direction in *Rosalina floridana* was affected by the nutrition and life-cycle, with gamontic generations being predominantly dextral while the agamonts being preferably left coiled. As, such morphological changes might possibly be attributed to climatic changes, laboratory culture studies helped to outline the effect of mode of reproduction on coiling direction of individuals of certain species.

I. Other factors affecting the abundance and morphology

Besides the studies discussed above, that refined the understanding of various factors which affect benthic foraminiferal diversity, abundance and morphology; laboratory culturing of benthic foraminifera also revealed certain other factors/processes that may influence the benthic foraminiferal characteristics as observed in the field. Myers (1942) observed that the large tests of *Elphidium crispum* evade destruction during the ingestion by larger macrofauna and calculated that ~1000 tests per square foot per year are contributed to the sediments. Reproduction occurred only during the beginning of March. An interesting finding was that the growth during different seasons can be differentiated on the basis of change in chamber morphology, thus providing a potential technique to infer the relative strength or duration of different seasons during the geologic past.

2.2.2.2. Studies that helped understand the factors affecting chemical composition

Though, stable isotopic and elemental composition of benthic foraminiferal tests has been developed as potential technique to infer past seawater temperature, salinity, productivity etc, a complete understanding of factors affecting the chemical composition of the tests is still lacking (Henderson, 2002; Lea, 2003). Thus laboratory culture studies have been carried out since long to understand the role of different physico-chemical parameters on the chemical composition of the foraminiferal tests. Much of the information about the effect of different physico-chemical parameters and vital factors on the foraminiferal chemical composition has been obtained by culturing benthic foraminifera under controlled laboratory conditions and has thus refined the paleoclimatic/paleoceanographic application of benthic foraminiferal chemical composition.

A. Studies carried out to understand the factors affecting stable oxygen and carbon isotopic composition of the tests

Though stable isotopic composition of foraminiferal test was considered as potential proxy for past seawater temperature, salinity, productivity and circulation changes depending upon the location and type of species, disequilibrium in the precipitation of $CaCO_3$ by benthic foraminifera, as compared to the ambient seawater, was noted by many workers (Duplessy *et al.,* 1970; Woodruff *et al.,* 1980). Thus laboratory culture studies under controlled physico-chemical conditions were carried out to understand the various factors that control the stable oxygen and carbon isotopic composition of benthic foraminifera.

Erez (1978) assigned the disequilibrium between foraminiferal and seawater carbon isotopic composition to the incorporation of lighter carbon resulting from the increased photosynthesis by symbiont algae. Not only the bottom water but also the pore water carbon isotopic composition significantly influences the carbon isotopic signal of the benthic foraminifera. But the carbon isotopic composition of benthic foraminifera is different than both the pore water and bottom water carbon isotopic composition (Chandler *et al.,* 1996). Wilson-Finelli *et al.* (1998) noted that the carbon isotopic signal of *Cibicidoides pachyderma* reflected the changing seawater chemistry to some extent. The oxygen isotopic composition of one of the most frequently used benthic foraminiferal species *Uvigerina peregrina* was found to be consistently

enriched relative to the medium, thus questioning the use of its oxygen isotopic composition for paleoceanographic studies. Similarly *Buliminella marginata* and *Discorbinella* sp. were not able to record the oxygen isotopic signal of the seawater at all. Williams *et al.* (1981) reported significant disequilibrium in carbon and oxygen isotopic fractionation in *Heterostegina depressa*, which changes as per the light intensity and age. As the species has symbionts, the disequilibrium was suggested as a result of exchange of carbon and oxygen during various metabolic activities of symbiont and host.

In comparison to planktic foraminifera limited studies have been carried out to understand the factors controlling the stable isotopic composition of the benthic foraminifera, probably because of the difficulty in maintaining the constant physico-chemical parameters throughout the duration of the experiment under laboratory conditions.

B. Studies carried out to understand the factors affecting elemental composition of benthic foraminiferal tests

Like the stable oxygen and carbon isotopic composition, the elemental ratios of the foraminiferal tests has also been established as potential proxies for paleoceanographic and paleoclimatic interpretation. Laboratory culture studies were carried out to understand the factors controlling the partitioning of these elements in the foraminiferal tests as well as to develop numerical relationship between physical parameter and concentration of element in the foraminiferal tests.

The Ba/Ca, Cd/Ca partitioning coefficient for *Bulimina marginata*, *Cibicidoides pachyderma* and *Uvigerina peregrina* under laboratory culture was nearly similar to that obtained from core-top calibrations in the field. However, large uncertainty was reported in Cd/Ca incorporation, probably reflecting the habitat effect. Huge water pressure does not play any role in the trace element partitioning in the benthic foraminifera (Havach *et al.*, 2001). Toyofuku *et al.* (2000) established the application of Mg/Ca of *Planoglabratella opercularis* (d'Orbigny) and *Quinqueloculina yabei* for paleotemperature reconstruction based on laboratory culture study. The salinity had insignificant effect on the Mg/Ca incorporation in the studied species.

However, laboratory culture experiments have not always been able to resolve the uncertainties as reported from the field. The uncertainty in the Mg, Sr, Cd, Ba versus Ca partitioning in *Bulimina aculeata* was as large or even more than that reported

from the field. Additionally the inter-individual variation was larger than the analytical precision. Inter-species variability was also observed as the partitioning coefficients for *Rosalina vilardeboana* were significantly different than that of *Bulimina aculeata* (Hintz *et al.,* 2006a). The ontogenetic development has element specific effect on the partitioning in benthic foraminifer. While the Sr/Ca partitioning co-efficient remained uniform throughout the different stages of development of *Bulimina aculeata*, large variation was observed in Mg/Ca partitioning co-efficient (Hintz *et al.,* 2006b). Maréchal-Abram *et al.* (2004) reported Cd/Ca partitioning coefficient value of 1 for *Ammonia beccarii* under one set of conditions. However deviation was reported in other sets and was attributed to the presence of foreign food material. In a recent study, de Nooijer *et al.* (2007) reported that the copper partitioning coefficient in two benthic foraminferal species was constant over a large range of seawater Cu/Ca concentrations and was not affected by the change in temperature and salinity of the seawater. As one of these species has symbionts, the similiarity in the copper partitioning co-effiecient of these two species showed that presence of symbionts does not affect the Cu partitioning in these species. The study has far reaching implications for the heavy metal pollution monitoring in coastal areas by using benthic foraminiferal elemental composition.

2.2.2.3. Studies that refined evolutionary position of foraminifera

An important application of benthic foraminifera is for correlation of oil bearing stratigraphic datums from different parts of the world. This application arises from the large geographic but short geologic range of benthic foraminiferal species. One application of molecular systematic analysis is the comparison of genetic make up of a group with others in order to find closeness or differences that in turn provide idea about the evolution of the group.

Pawlowski *et al.* (1994) based on the sequencing of large subunit rDNA of four benthic species (*Ammonia tepida, Rosalina vilardeboana, Glabratella erecta, and Trochammina* sp.), inferred that the foraminifera evolved along with the plasmodial and cellular slime molds, much earlier than that suggested by the fossil record. However, in a subsequent study, based on the molecular systematic analysis of *Ammonia* sp., Wray *et al.* (1995) showed that the foraminifera originated from heterokaryotic flagellated marine protist, probably sometime in the later Proterozoic. The findings were in contrast to the previously held belief that foraminifera originated

from amoeba like ancestor. Later on Pawlowski *et al.* (1996) confirmed the early foraminiferal evolution among the eukaryotic group, based on the sequencing of small sub unit rDNA genes of three species of foraminifera (*Ammonia beccarii, Trochammina* sp. and *Allogromia* sp.) and suggested that Wray *et al.* (1995) attributed a wrong sequence to foraminifera. Later on close similarity in the partial sequences of 3' end of the small subunit rRNA gene (SSU rDNA) in 22 different species belonging to all major foraminiferal groups lead to the conclusion that foraminifera are monophyletic in origin (Pawlowski *et al.,* 1997). The studies have significantly refined the evolutionary status of foraminifera and thus their application for stratigraphic correlation studies.

2.2.2.4. Studies that helped to identify cryptic species

Species level identification is the basic requirement for almost all foraminiferal characteristics based proxies for paleoclimatic/paleoceanographic reconstruction. Significant differences are reported in response of different species belonging to same genus, to the physico-chemical parameters, as has been discussed above. Marked differences have also been noted in the preferred microhabitat of species. Thus efficient application of foraminiferal characteristics to environmental conditions during geologic past requires that the species be identified properly.

As discussed above numerous attempts were made to resolve the slight morphological changes in certain individuals through laboratory maintenance of species throughout the life-cycle, in order to help in species identification. In an interesting study Arnold (1952) observed that a particular structure in *Grovia oviformis* sustains desiccation and thus can be used as an aid in identification of the species from fossil assemblages. The morphological differences in gamonts and agamonts of a species, as well as within gamonts and agamonts of same species (*Allogromia laticolaris*) after several generations were observed by Arnold (1953). It paved the way for clubbing of previously different species into single species. However, Röttger *et al.* (1986) and Röttger (1987) based on laboratory observations, proposed that the so called schizonts of *Heterostegina depressa* were in fact a new species. But the hypothesis was later retracted (Röttger *et al.,* 1990a). Pawlowski & Lee (1992) also used laboratory culturing to identify morphologically different gamonts and shizonts for many species, which otherwise may be considered as two different species. In a contrasting study previously considered separate species of *Cibicides* were found to be different

ontogenetic stages of *Cibicides lobatulus*. In a similar study Röttger *et al.* (1990b) identified morphological differences, including the number of chambers, test size and size of the proloculus, between gamont and schizonts of *Calcarina gaudichaudii*. Fahrni *et al.* (1997) used the immunoblotting of actin to suggest that *Miliammina fusca* belong to porcellaneous rather than agglutinated foraminifera.

Except for the apparent morphological changes observed between the different ontogenetic stages of the same species, molecular systematic analysis of benthic foraminifera has recently been started to identify cryptic species. The genetic make-up of each individual is characteristic. The differences increase with the increasing taxonomic level. It thus helps in identification of species or genus, based on the parentage of differences in the genetic make-up of the individuals. The extraction and enzymatic characterization of foraminiferal DNA lead the way for application of molecular systematic analysis for identification of foraminiferal species and for tracing the evolutionary history of foraminifera (Langer *et al.*, 1993; Wray *et al.*, 1993). Pawlowski *et al.* (1994) showed potential application of molecular systematic analysis techniques for species identification and grouped eight different morphotyes of *Glabratella* in two different species. Pawlowski *et al.* (1995) though found distinct differences in the partial sequences of large subunit ribosomal DNA of all six morphotypes of *Ammonia,* collected from the field, identified three distinct species of *Ammonia*. Later on Holzmann & Pawlowski (1997) identified two distinct species of *Ammonia* based on the molecular analysis and also recognized morphological and ecological differences among these two species. Tsuchiya *et al.* (2000) used ribosomal DNA analysis technique to infer the status of different morphotypes of *Glabratellidae* and observed that the *Glabratellidae* specimens were genetically less diverse as compared to the *Ammonia* specimens. By using sequences of internal transcribed spacers of ribosomal DNA, Tsuchiya *et al.* (2003) identified two different species of *Planoglabratella opercularis*, and later on supplemented the findings of molecular analysis, with geographic distribution of the species. Thus the molecular systematic analysis technique can be effectively used to differentiate between morphotypes of benthic foraminifera.

Though the molecular systematic analysis helped to identify distinct cryptic species from morphotypes of the same species and these species could be identified based on morphological differences, Holzmann *et al.* (1998) identified two *Ammonia* species based on the differences in the partial sequencing of large subunit ribosomal DNA,

but the species could not be identified morphologically. Similarly, Pawlowski *et al.* (2002) identified 49 Allogromid species belonging to 28 genera/families, from the 27 morphotypes collected from the Antarctic waters. In an interesting study Grimm *et al.* (2007) recovered genetic material of entirely different non-calcareous taxa from the *Chilostomella* tests, suggesting that it may lead to wrong identification of the tests as belonging to *Chilostomella* during live-staining techniques. The studies have far reaching implications for species identification in foraminifera.

2.2.2.5. Other studies that refined application of benthic foraminifera for past climatic/oceanographic reconstruction

Except the studies mentioned above, during the course of laboratory culture of benthic foraminifera, certain other factors have also been found to influence the foraminiferal abundance and morphology and leave a perceivable effect on faunal composition. Frankel (1974) observed that *Trochammia ochracea* occupies the cavities in the sediment surface and may be missed during the counting of foraminifera. Kuile & Erez (1991) proposed the presence of internal carbon pool for calcification and partial contribution to this internal carbon pool by the organic matter respiration. However, the amount of carbon contributed by the organic matter respiration to the internal carbon pool varied from species to species. Moodley *et al.* (1998a) observed the adverse effect of sulphidic conditions on the foraminiferal density and reproduction. Though the foraminifers could tolerate short-term exposure to sulphidic conditions, persistent exposure lead to significant decline in the foraminiferal density and almost complete cessation of reproductive activity. Studies carried out to understand the substrate preference of benthic foraminifera did not give encouraging results as Kitazato (1995) observed that faunal density in the artificial substrates was order of magnitude lower than the normal field conditions and that too most of the species were confined to the freshly naturally deposited, few cm thick sediment layer overlying the artificial substrate.

2.2.3. Conclusions

After reviewing the benthic foraminiferal culture studies it is noticed that-

- Most of the studies were done by biologists who were interested in studying the soft part of foraminifera

- Studies dealing with the hard part of foraminifera were comparatively less and the proper understanding of the parameters affecting the morphology of the benthic foraminifera is still lacking.
- Exact mechanism that leads to deformities in the benthic foraminiferal tests is yet to be explored.
- Efforts are required to understand the specific effect of different pollutants, so that the benthic foraminiferal response can be used efficiently in the field.
- Factors affecting the foraminiferal isotopic composition, especially the carbon isotopic composition are still not known fully.

2.3. State of art: National

The state of art of laboratory culture studies on benthic foraminifera as evident from the review of nearly 162 manuscripts indicate that - Though popular in other countries, laboratory studies on foraminifera are yet to attain proper attention in India, the only representation is from 'Foraminiferal Culture Laboratory', National Institute of Oceanography, Goa making it the sole laboratory dealing with this aspect foraminiferal research in this part of the world. Table 2.2 summarizes the research work on experimental studies on benthic foraminifera from India with a brief description on the study details of each research work.

Less than 2 decades since marking the beginning, Foraminiferal culture studies in India is yet to catch the attention of more researchers from the field. Addressing the need of foraminiferal culture studies to refine the field based findings based on foraminifera (Nigam *et al.*, 1996a) the first results on foraminiferal culture experiments was published in the year 1996 (Nigam *et al.* 1996b) where they monitored the effect of different media and food on the growth of benthic foraminifera *Rosalina leei.* The results suggest that the Erdscheriber medium was conducive for the general growth of *Rosalina leei* and food was found to govern their growth in sea water.

Khare & Nigam (2000) studied the rate of movement of benthic foraminifer *Quinqueloculina* reported to vary 0.034 to 0.139mm/min. In an attempt to explore the relation between the molecular properties of protoplasm and the morphological variations in benthic foraminifera, the role of 12 S mitochondrial gene on dimorphism and coiling direction in *Pararotalia nipponica* was studied (Saraswat *et al.,* 2003).

The response of benthic foraminifera to varying oxygen concentrations revealed that any change from the normal oxygen concentrations- high or low, will lower the foraminiferal numbers of that region (Panchang *et al.*, 2006). Making efforts to refine the foramiminiferal proxies for paleoclimate and marine pollution monitoring, different studies were carried out. The *P.niponica* specimens were subjected to different salinity conditions and their response showed that 35‰ salinity was the most suitable for their growth; the lower as well as higher salinities showed lesser growth in the living specimens (Nigam *et al.*, 2006a). In a further attempt, Nigam *et al.* (2008) studied the combined effect of salinity and temperature on benthic foraminiferal species *Rosalina leei.* The results showed that 25°C temperature and 35‰ salinity were most suitable for the growth of *Rosalina leei.* Results are significant as the benthic foraminiferal response to different salinity and temperature are being used for paleoclimatic reconstruction.

In order to develop efficient foraminiferal proxies for heavy metal pollution studies, experimental studies were conducted on benthic foraminiferal species *Rosalina leei.* Juvenile specimens of *R. leei* were subjected to gradual increase in mercury concentration (Saraswat *et al.*, 2004) and observations were again confirmed and compared with the sudden additions of mercury concentrations in to the media of juvenile *R. leei* specimens (Nigam *et al.*, 2009) and the results show that the response of benthic foraminifera *R.leei* is different and distinct to gradual and sudden stress conditions, but the type of abnormalities observed remain similar. The results are very relevant to characterize the response of *R. leei* to heavy metal mercury

The successful application of foraminiferal characteristics to infer the past climatic/ oceanographic reconstructions and marine pollution studies make them a widely accepted and practically applied proxy compared to other microorganisms. The experimental studies on foraminifera help to refine the difference in the foraminiferal characteristics from physico-chemically different environments as observed in the field. However, despite a large number of culture studies being carried out on benthic foraminifera with their vivid application in focus, still much more efforts are needed to understand the parameters affecting the benthic foraminiferal abundance, morphology and chemical composition. More efforts are needed from the Indian subcontinent towards this direction considering the vast gap between the state of art of the experimental studies on foraminifera in the International and National scenario.

Table 2.1: Manuscripts on the experimental studies on benthic foraminifera: International Scenario

Sl. No.	Author & Year of Publication	Study details
1	Myers (1935a)	The optimum temperature for *Patellina corrugata* is very near to the upper temperature tolerance limit; lower temperature leads to decreased rate of reproduction.
2	Myers (1936)	Close relationship between mode of reproduction and coiling direction in *Spirillina vivipara.*
3	Myers (1940)	Megalospheric specimens preferentially coiled sinistrally while the microspheric ones coiled dextrally in *Discorbis patelliformi*
4	Myers (1942)	About 1000 *Elphidium crispum* tests per square foot are contributed annually to the sediments; larger tests evade degradation during ingestion by macrofauna.
5	Arnold (1952)	Desiccation experiments showed that a part of tubuliferous ring may get preserved after fossilization and help in identification from fossil assemblage.
6	Arnold (1953)	Observed live specimens of *Allogromia laticolaris* in the laboratory and inferred morphological differences in gamonts and agamonts; helped in clubbing of previously different species under single species.
7	Arnold (1954a)	*Discorinopsis aguayi* and *Discorinopsis vadesens* can survive extremes of temperature only if subjected for a short period.
8	Bradshaw (1955)	Different rotalid species have different food preferences; comparatively lower temperature results in reduced growth rate; both higher and lower than normal salinity has adverse effect on the growth of rotalids.
9	Bradshaw (1957)	Temperature and salinity below and above tolerance limits, lead to cessation of growth in *Streblus beccarii* (Linné); within the temperature tolerance limit, growth increases with temperature; lower temperature and extreme salinity leads to delayed reproduction.
10	Nyholm (1958)	Observed that previously considered separate taxa were in fact different ontogenetic stages of *Cibicides lobatulus.*
11	Bradshaw (1961)	Higher temperature lead to the increased growth rate and quick reproduction; effect of temperature and pH on benthic foraminifera was linked with seawater salinity; scarcity of food lead to the decreased growth and reproduction; oxygen consumption was species specific and was controlled by the seawater temperature.
12	Lee *et al.* (1961)	The response of benthic foraminifera to a combined diet of diatom, filamentous algae and bacteria varied from species to species.
13	Freudenthal *et al.* (1963)	Developed a tidal system for laboratory studies on eulittoral foraminifera and found that the higher salinity is correlated with early reproduction.
14	Lee *et al.* (1963)	Close link between mode of reproduction and coiling direction with gamont specimens being preferentially dextrally coiled while the agamonts being sinistral.
15	Murray (1963)	Performed various ecologic experiments on foraminifera.
16	Sliter (1965)	Laboratory experiments on the lifecycle and ecologic controls of *Rosalina globularis* d'Orbigny.
17	Angell (1967)	*Rosalina floridana* can recover the dissolution of the tests incurred while subjected to seawater with acidic pH; however no evidence of any special mechanism to regenerate the test.
18	Schnitker (1967)	At sexual maturity under laboratory culture, offsprings of *Triloculina linneiana* attained only the one-eighth the size of the parent and were morphologically distinctly different than the parent.
19	McEnery & Lee (1970)	Incorporation of radionuclides of Ca, Sr, P and S was proportional to the growth rate of *Rosalina leei* and *Spiroloculina hyalina*; both the species have the capability to regenerate the test.
20	Röttger (1972b)	Low intensity and darkness lead to cessation of growth activity; growth pattern changed after the specimens were subjected to normal light again. Lower temperature resulted in reduced rate of chamber formation but does not affect the size of chambers or shape of the test.

Sl. No.	Author & Year of Publication	Study details
21	Röttger & Berger (1972)	Very high light intensity lead to decreased growth rate and tests grown under such conditions were morphologically distinct.
22	Lee & Muller (1973)	*Allogromia laticollaris, Rosalina leei*, and *Spiroloculina hyalina* are selective feeders and can adjust to the seasonal changes in the food availability.
23	Frankel (1974)	*Trochammina ochracea* attaches to the cavities in the surfaces and may be missed during counting.
24	Schnitker (1974)	Mature and nearly mature specimens are incapable of adjusting to different temperature regimes; physiological acclimatization takes place during and possibly after reproduction over a span of generation; clone culture lead to morphological variations.
25	Muller (1975)	Assessed temperature, salinity and pH limits for *A. laticollaris, R. leei* and *S. hyalina*; type and amount of food affect the food intake.
26	Lee & Bock (1976)	Two species of symbiont bearing soritid foraminifera, added about 4% of their weight in additional calcium each day; light did not enhance the rate of calcification.
27	LeFurgey & St. Jean (1976)	Species diversity was approximately 20% higher and average number of living foraminifera was approximately 5 times greater in control ponds than in the effluent ponds.
28	Röttger & Spindler (1976)	Studied the optimum condition for growth of *H. depressa* including the light intensity and the symbiotic algae; described the embryonic and nepionic developmental stages of the living individuals.
29	Salami (1976)	Feed preference optimum for growth and reproduction (salinity range and temperature range), mode of reproduction, chamber addition, differences in size, number and arrangement of nuclei etc of *Trochammina cf. T. quadriloba*
30	Ross (1977)	Size of the animal depends on the composition of food available; reproduction is adapted to seasonal changes in food.
31	Schwab (1977)	Described the biology (size, colouration, behaviour and the ability to rapidly change shape) of a new foraminifera belonging to genus *Boderia* and family Lagynidae.
32	Erez (1978)	Carbon isotopic composition of the foraminifera becomes more depleted under increased rate of photosynthesis by attached symbiont algae.
33	Hallock (1978)	Studied the rate of carbon fixation by *Amphistegina lessonii* (in the light as well as in dark) and noted that carbon fixation in light was consistently higher than that in the dark.
34	Rottger (1978)	Unusual multiple fission in the gamont of the larger foraminifera *H. depressa*; observed regeneration following multiple fission during which a small residue of protoplasm remained within the vacated test.
35	Angell (1979)	Studied the crystal growth during chamber development in *R. floridana*; also studied the calcium and carbonate uptake during the same developmental periods using tracer method.
36	Lee *et al.* (1979)	The symbiosis is responsible for the comparatively larger size of the symbiont bearing benthic foraminifera.
37	Lopez (1979)	The food intake varies as per the density of chloroplast; the chloroplast abundance varies as per changing light-dark conditions.
38	Lee (1979)	Nutrition and physiology of foraminifera from littoral, sub littoral to temperate zones is discussed.
39	Lee *et al.* (1980)	*A. hemprichii* and *A. lobifera* were photoinhibited above 200 klx illumination, while photoinhibition in *Amphistegina lessonii* and *Heterostegina depressa* occurred at lower than 10 klx light intensity.
40	Hallock (1981)	Effect of light on growth rates of *A. lessoni* and *A. lobifera* are studied in the laboratory as well as the field conditions.
41	McEnery & Lee (1981)	Three species of larger foraminifera *A.lobifera, A.hemprichii and H. depressa* were studied for their endosymbiotic associations and also fine structure analysis.

Sl. No.	Author & Year of Publication	Study details
42	Williams *et al.* (1981)	Identified significant disequilibrium in carbon and oxygen, isotopic fractionation in *Heterostegina depressa* and attributed it to the vital effects that varied with changing light intensity and age.
43	Duguay (1983)	Calcium incorporation and photosynthetic carbon fixation was examined in three species of benthic foraminifera that harbor symbiotic microalgae.
44	Zimmerman *et al.* (1983)	*Heterostegina depressa* tests, secreted at increased light levels are depleted in heavier isotopes of oxygen and carbon, probably under the influence of symbiont photosynthetic activity.
45	Bowser & Bloodgood (1984)	Phase-contrast light microscopy and membrane surface marker studies on two protozoan systems including that of *A. laticollaris* and *Ammonia* sp. indicated that surface motility does not occur by a surf- riding/surf boarding mechanism.
46	Kuile & Erez (1984)	Growth rate decreases under dark conditions in symbiont bearing foraminifera; shell thickening occurs under turbulent conditions.
47	Biekart *et al.* (1985)	Existence of two biologically different types of megalospheric *H.depressa* in laboratory cultures as well as Hawaiian sediments.
48	Delaney *et al.* (1985)	Temperature dependence for the minor elemental composition of foraminiferal shell was investigated in the laboratory for one benthic foraminiferal species
49	Hallock *et al.* (1986)	Influence of environment, especially availability of light and water motion on the test shape of *Amphistegina*.
50	Röttger *et al.* (1986)	Proposed that the earlier considered schizont of *Heterostegina depressa*, in fact belonged to a new species.
51	Kuile & Erez (1987)	Uptake of inorganic carbon and internal carbon cycling in symbiont bearing benthonic foraminifera.
52	Kuile *et al.* (1987)	Attempted to define the role of feeding in the carbon metabolism of the host-symbiont system in larger symbiont bearing foraminifera.
53	Röttger (1987)	Describes and illustrates a new species of *Heterostegina* (*H.apogama*,n.sp.) based on the reproductive as well as morphological characteristics.
54	Goldstein (1988)	Reported the alternation of generations in the life cycle of *Saccammina alba* Hedley.
55	Grell (1988)	Reported the extreme heteromorphy in the alternative generations of monothalamous foraminifera *Heterotheca lobata*.
56	Kuile & Erez (1988)	Established the existence of an internal inorganic carbon pool in the perforate foraminifer *Amphistegina lobifera* using ^{14}C tracer method.
57	Buzas (1989)	No effect of mineralogically different sediments on the colonization potential of foraminifera.
58	Buzas *et al.* (1989)	Conducted experiments on predation, substrate preference and colonization of benthic foraminifera at the Shelf break off the Ft. Pierce inlet, Florida.
59	Röttger *et al.* (1990b)	The gamonts and schizonts of *Calcarina gaudichaudii* vary in their test size, size of the proloculus and number of chambers.
60	Röttger *et al.* (1990a)	Observed the formation of megalospheric schizonts by a microspheric agamont for the first time, which verifies part of the hypothesis of biologic trimorphism.
61	Boltovskoy (1991)	Conducted laboratory experiments to study the destruction of foraminiferal tests.
62	Faber & Lee (1991)	Studied the effect of feeding on the growth of foraminifera.
63	Kuile & Erez (1991)	Presence of internal carbon pool for calcification and partial contribution of carbon to this pool from the organic matter respiration.
64	Lee *et al.* (1991)	Response to food was species specific as few species grew more when fed while others showed increased growth when provided with no food; symbiont bearing species could not survive prolonged darkness; additional nitrate and phosphate does not change the growth rate under certain conditions.

Sl. No.	Author & Year of Publication	Study details
65	Linke (1992)	Two survival strategies in benthic foraminifera based on the ATP content and metabolic rates, namely, the one that maintained uniform rate throughout the year, and those that showed seasonally varying ATP turnover rate.
66	Pawlowski & Lee (1992)	Showed that *Rotaliella elatiana* has a classical heterophasic lifecycle, with a regular alternation of diploid agamontic phase and a haploid gamontic phase.
67	Bernhard (1993)	No evident statistically significant effect of changing oxygen condition on the survival or ATP pool of foraminifera.
68	Goldstein & Moodley (1993)	Life cycle of *Ammonia becarii forma tepida* includes both sexual and asexual phases and is probably best characterized as a facultative alternation of generations.
69	Wray *et al.* (1993)	Discussed the extraction and enzymatic characterization of foraminiferal DNA.
70	Chandler *et al.* (1994)	Utility of sediment microcosm cultures for the study of ontogenetic and sediment microhabitat effects on isotopic composition of benthic foraminifera.
71	Goldstein & Corliss (1994)	Organic detritus, associated sediments as well as bacterial cells act as food for deep-sea and shallow-water species.
72	Pawlowski *et al.* (1994a)	Identified two species of *Glabratella* from eight morphotypes collected from the field, by using molecular systematic analysis.
73	Pawlowski *et al.* (1994b)	Inferred that foraminifera branch close to the plasmodial and cellular slime molds in the eukaryotic evolutionary tree, which is in contrast to the fossil records.
74	Alve & Bernhard (1995)	Studied the vertical migratory response of benthic foraminifera to controlled oxygen concentrations ranging from well-oxygenated to dysaerobic conditions in experimental mesocosm.
75	Bresler & Yanko (1995)	Presence of unidentified organic compounds decreased the toxic effect of heavy metals on the *Pararotalia spinigera* and *Rosalina macropora*; potential application of foraminifera for pollution monitoring owing to their response to xenobiotics.
76	Hemleben & Kitazato (1995)	The culture without food survived for longer duration but reproduced less than the ones maintained under continuous food supply.
77	Kitazato (1995)	*In situ* recolonization experiments to understand the possible substrate preferences of deep sea benthic foraminifera; faunal density was much lower and mainly confined to the natural sediment deposited over the artificial substrate.
78	Linke *et al.* (1995)	The response of deep sea benthic foraminifera to a simulated sedimentation was assessed in a strip-board microorganism by using TEM (Transmission Electron Microscopy) Organic Carbon, Adinosine Nucleotide, ETS assay and line observation.
79	Pawlowski *et al.* (1995)	Identified three species of *Ammonia* based on the partial sequences of large subunit ribosomal DNA from six morphotypes of *Ammonia*; all the six morphotypes had distinct LSU rDNA.
80	Wray *et al.* (1995)	Concluded that the foraminifera were derived from a heterokaryotic flagellated marine protist, probably sometime in the later Proterozoic, based on the cytological features and the DNA sequences of *Ammonia*.
81	Bernhard & Alve (1996)	Survival rate of *Adercotryma glomeratum*, *Psammosphueru bowmunni* and *S. fusiformis* subjected to anoxic conditions does not vary much from the control specimens; however the ATP concentrations were significantly lower. *B. marginata* behaved differently.
82	Chandler *et al.* (1996)	Sediment microhabitat effects on carbon isotopic signatures of microcosm-cultured benthic foraminifera *Ammonia beccarii*.
83	Holzmann *et al.* (1996)	Sequence variations in the large- subunit ribosomal RNA gene of *Ammonia* (foraminifera, Protozoa) and their evolutionary implications.

Sl. No.	Author & Year of Publication	Study details
84	Pawlowski *et al.* (1996)	Foraminifera were probably the first eukaryotes to branch off from the main eukaryotic tree.
85	Fahrni *et al.* (1997)	*Miliammina fusca* is porcellaneous and not agglutinated as suggested before.
86	Holzmann & Pawlowski (1997)	Identified two species of *Ammonia* based on the molecular, morphological and ecological evidence.
87	Pawlowski *et al.* (1997)	Confirmed monophyletic origin of foraminifera.
88	Holzmann *et al.* (1998)	Identified two *Ammonia* sp. based on the differences in the partial sequencing of large subunit ribosomal DNA, but the species could not be identified morphologically.
89	Moodley *et al.* (1998b)	Few foraminifera can survive under anaerobic conditions; soft-shelled foraminifera are less tolerant to anoxia.
90	Moodley *et al.* (1998a)	Sulphidic conditions resulted in significant reduction of foraminiferal density; none of the species reproduced under sulphidic conditions.
91	Wilson-Finelli *et al.* (1998)	While *Cibicides pachyderma* incorporated the changing carbon isotopic signal of the medium, *Uvigerina peregrina* ^{18}O was relatively enriched, in contrast to the field studies.
92	Alve & Olsgard (1999)	Extreme copper contaminated sediments does not defer foraminifera from colonization; no correlation between copper contamination and abundance of abnormal specimens.
93	Stouff *et al.* (1999b)	Increased number of abnormal *Ammonia beccarii* and *Ammonia tepida* specimens under hypersaline conditions; abnormalities similar to those reported from similar environments in field.
94	Stouff *et al.* (1999a)	Though abnormal specimens were also present under normal conditions, hypersaline conditions lead to the increased abundance of abnormal specimens.
95	Gross (2000)	Species-specific effect of change in temperature, oxygen and food quantity on the migrational activity.
96	Gustafsson *et al.* (2000)	Introduction of small amount of Tri-n-Butyltin lead to increased population density, probably due to reduced predation; higher concentration had adverse effect.
97	Holzmann (2000)	Deciphered the subspecies of *Ammonia* through molecular systematics.
98	Moodley *et al.* (2000)	*Ammonia* responded best to the freshly added phytodetritus.
99	Nigam & Caron (2000)	Reported a direct relationship between the pairing in *R.leei* and temperature
100	Toyofuku *et al.* (2000)	Monospecific Mg/Ca can be used to infer paleotemperature, based on culture of *Planoglabratella opercularis* (d'Orbigny) and *Quinqueloculina yabei* Asano under controlled conditions; salinity does not have significant effect on the foraminiferal Mg/Ca.
101	Tsuchiya *et al.* (2000)	Ribosomal DNA sequence studies show that the single specimens of *Glabratellidae* are genetically less diverse as compared to *Ammonia.*
102	Havach *et al.* (2001)	Ba/Ca and Cd/Ca partitioning coefficients of *Bulimina marginata, Cibicidoides pachyderma and Uvigerina peregrina* were within the range observed from the field.
103	Heinz *et al.* (2001)	Benthic foraminiferal abundance increased under increased food supply and oxygen; the within sediment migration was controlled by the availability of oxygen.
104	Heinz *et al.* (2002)	Time series experiment to investigate the response of cultures deep sea benthic foraminifera to simulated phytodetritus pulses under stable oxygen concentrations.
105	Moodley *et al.* (2002)	Differential response of benthic foraminifera to induced phytodetritus.
106	Pawlowski *et al.* (2002)	Identified 49 Allogromids species belonging to 28 genera/families, from the 27 morphotypes, collected from Explorers Cove, McMurdo Sound, Antarctica, based on 135 partial small-subunit ribosomal DNA sequences.

Sl. No.	Author & Year of Publication	Study details
107	Cadre *et al.* (2003)	Temporary acidification of the environment can cause morphological abnormalities in the *Ammonia beccarii* foraminiferal tests during recalcification.
108	Duijnstee *et al.* (2003)	Anoxic conditions lead to the comparatively shallower dwelling depth for most of the species.
109	Tsuchiya *et al.* (2003)	Identified two different species of *Planoglabratella opercularis* by sequencing internal transcribed spacers of ribosomal DNA.
110	Ward *et al.* (2003)	*Haynesina germanica* did not consume sewage derived particulate organic matter; it might have fed only bacteria associated with sewage POM.
111	Witte *et al.* (2003)	Abyssal foraminiferal response to phytodetritus was delayed and distinct from continental slope foraminifera.
112	Fujita (2004)	Field colonization experiment using artificial substrate was conducted to examine the small scale distribution of algal symbiont bearing larger foraminifera on reef rubble.
113	Geslin *et al.* (2004)	*Globobulimina affinis, Hoeglundina elegans, Pyrgo murrhina, Uvigerina peregrina, Uvigerina mediterranea* can all live in the oxic sediment layer, whereas *G. affinis* can also live under anoxic conditions; oxygen concentration regulates the microhabitat.
114	Maréchal-Abram *et al.* (2004)	Reported cadmium partitioning coefficient value close to one for *A.beccarii* under one set of conditions, and opined that cadmium does not segregate in the foraminiferal carbonate from the surrounding water
115	Williams & Hallock (2004)	The growth rate of *Amphistegina* increased in blue light while was not affected by ultra-violet light towards the lower end.
116	Ernst *et al.* (2005)	Though oxygen availability affected the short term vertical distribution and density of benthic foraminifera, food content was responsible for shaping the long-term benthic foraminiferal assemblages.
117	Langezaal *et al.* (2005)	*Allogromia laticollaris* and *Ammonia beccarii* could distinguish between food (living and dead bacteria) and non-food (inorganic particles) material; inter- and intra specific variation in the uptake rate and final digestion of food.
118	Nomaki *et al.* (2005a)	Shallow infaunal species (*Uvigerina akitaensis, Bulimina aculeata*) assimilated more carbon as compared to the intermediate (*Textularia kattegatensis*) and deep infaunal species (*Chilostomella ovoidea*); response varied as per the food and season.
119	Nomaki *et al.* (2005b)	Vertical migration in response to addition of food; response decreased from shallow infaunal to deep infaunal species; *Chilostomella ovoidea* does not respond at all.
120	Cadre & Debenay (2006)	Copper contamination resulted in delayed chamber formation and reproduction, thus reducing the abundance of two species of *Ammonia*; abnormal tests increased in number.
121	Ernst *et al.* (2006)	Faunal density decreased significantly in oil induced microcosms; few species reproduced quickly under polluted environment.
122	Hintz *et al.* (2006a)	Sr/Ca partitioning coefficient of *Bulimina aculeata* was not affected by the ontogenetic development, whereas large variation was observed in Mg/Ca partitioning between different individuals, as well as at different developmental stages in the same individual.
123	Nomaki *et al.* (2006)	Recognized three types of food preference, viz. (1) fresh phytodetritus selectively (phytophagous species); (2) fresh phytodetritus selectively but sedimentary organic matter as well when phytodetritus is absent; and (3) sedimentary organic matter at random (deposit feeders).
124	Topping *et al.* (2006)	No direct effect of sewage-derived particulate organic matter (POM) on the food sources and diets of *Ammonia beccarii* and *Haynesina germanica*; sewage POM was not used as food source.
125	de Nooijer *et al.* (2007)	Copper partitioning coefficient was constant in *Ammonia tepida* and symbiont-bearing *Heterostegina depressa* over a large range of seawater Cu/Ca and was not affected by the seawater salinity, temperature or presence of symbionts.

Sl. No.	Author & Year of Publication	Study details
126	Grimm *et al.* (2007)	Reported highly divergent genotypes among *Chilostomella* collected from different geographical locations, based on sequencing of 3′ region of the small subunit ribosomal RNA (SSU rDNA), 5.8 subunit and the internal transcribed spacers.
127	Kohoa *et al.* (2008)	Reported that benthic foraminifera respond to deposition of phytodetritus, either directly or indirectly due to enhanced bacterial activity; the response can be measured as an increase in TSS of foraminifera.
128	Pascal *et al.* (2008)	Grazing experiments were performed in order to measure effects of abiotic (temperature, salinity and irradiance) and biotic (bacterial and algal abundances) factors on uptake rates of bacteria.
129	Rollion-Bard *et al.* (2008)	Reported that the "vital effect" for oxygen isotopic composition in foraminifera is mainly due to the presence of primary calcite, which is at least 2–3& lighter than the secondary calcite.
130	Kuroyanagi *et al.* (2009)	Growth rate, measured by shell diameter, shell weight, and the number of chambers added, generally decreased with lowering pH after 10 weeks of culture in asexually produced individuals of *Marginopora kudakajimensis*.
131	Nomaki *et al.* (2009)	In situ feeding experiment using 13C-labeled unicellular algae, showed microbial degradation of 13C-labeled algal material and the production of bacterial biomass within 2 days. The biomass produced was gradually turned over by respiration or predation within 6 days.
132	Pucci *et al.* (2009)	Experiment results show that all dominant foraminiferal taxa from the sixteen short sediment cores from a 35 m deep site in the Adriatic Sea survive strongly hypoxic conditions.
133	Alve & Goldstein (2010)	Experiments on Skagerrak basin sediments show that propagules of certain species (<32 μm in size) are sufficiently resilient to survive transport, remain "dormant" for two years, and then start growing and reproducing once conditions permitted.
134	Dissard *et al.* (2010)	Magnesium incorporation increases with increasing temperature (DMg increases by 4.1%, 4.6%, and 5.5% per _C (temperature range 10–15 _C for salinities of 20, 33, and 40 psu, respectively), but to a lesser extent than previously described for other low-Mg calcite foraminifera.

Table 2.2: Manuscripts on the experimental studies on benthic foraminifera: National Scenario

Sl. No.	Author & Year of Publication	Study details
1	Nigam *et al.* (1996a)	Need of culture studies on benthic foraminifera
2	Nigam *et al.* (1996b)	Initial results of culture studies from Off Goa region
3	Khare & Nigam (2000)	Rate of movement in cultured benthic foraminifera
4	Saraswat *et al.* (2003)	Genetic studies on benthic foraminifera *Pararotalia nipponica*
5	Saraswat *et al.* (2004)	Response of *Rosalina leei* to mercury; benthic foraminifera as indicators of pollution
6	Nigam *et al.* (2006a)	Effect of salinity variations on *Pararotalia nipponica*
7	Panchang *et al.* (2006)	Effect of Oxygen manipulations on benthic foraminifera
8	Linshy *et al.* (2007)	Review of laboratory culture studies on benthic foraminifera
9	Nigam *et al.* (2008)	Response of *Rosalina leei* to different salinity temperature conditions
10	Nigam *et al.* (2009)	Effect of sudden stress due to heavy metal mercury on benthic foraminifera *Rosalina leei*

2.4. Objectives of the present work

Taking these facts in to account, urging the need of experimental studies and having very keen interest to attempt something interesting in the laboratory studies of foraminifera, this research work was taken up with the following objectives.

- To establish culture program and to observe life span and mode of reproduction of selective foraminiferal species
- To observe the response of benthic foraminifera to specific pollutants
- To characterize type of abnormalities (if any) in benthic foraminifera as a result of different pollutants

Since this is the first attempt of its kind from our country, maximum effort and dedication is paid towards establishing this particular branch of research and also to put a sustainable research background for the future researchers to explore the possibilities in the days to come. The basic methods involved are explained in the following chapter.

CHAPTER 3

MATERIALS AND METHODOLOGY

Chapter 3

Materials and methodology

"Inspiration usually comes during work, rather than before it."

- Madeleine L'Engle

3.1. Introduction

To achieve the goals of the present study, a number of field and laboratory procedures were adapted and are described in the following sections chronologically. An attempt is made to summarize these procedures right from the collection of samples to analyse in the laboratory in this chapter under two broad subdivisions.

- Field methods: involve the description of the areas from which samples have been collected for the present study, details of the procurement of samples and the steps involved in the initial treatment of samples in the field.
- Laboratory methods: involve the storage of samples, sorting of live foraminifera and maintenance of the living cultures under laboratory conditions.

The present work is a compilation of different experiments on living benthic foraminifera conducted under laboratory conditions. This includes the research work carried over in Foraminiferal Culture Laboratory, National Institute of Oceanography Goa, India and the work carried out in the Micropaleontology Laboratory, Tubingen University, Germany as a part of UGC- DAAD fellowship.

Since each experiment is having a different set-up based on the set objectives of the study, the laboratory preparation of samples and the experimental set-ups widely vary from each other; hence they are discussed in the respective chapters to get a the clear understanding of the steps involved in each study.

The procedures/methods adopted for the present study is summarized in a flow chart (Fig. 3.1). This flowchart gives a broad idea of the entire setup of this thesis. The details of the sampling stations and the different experiments conducted using sample from each location are clearly illustrated in the flowchart.

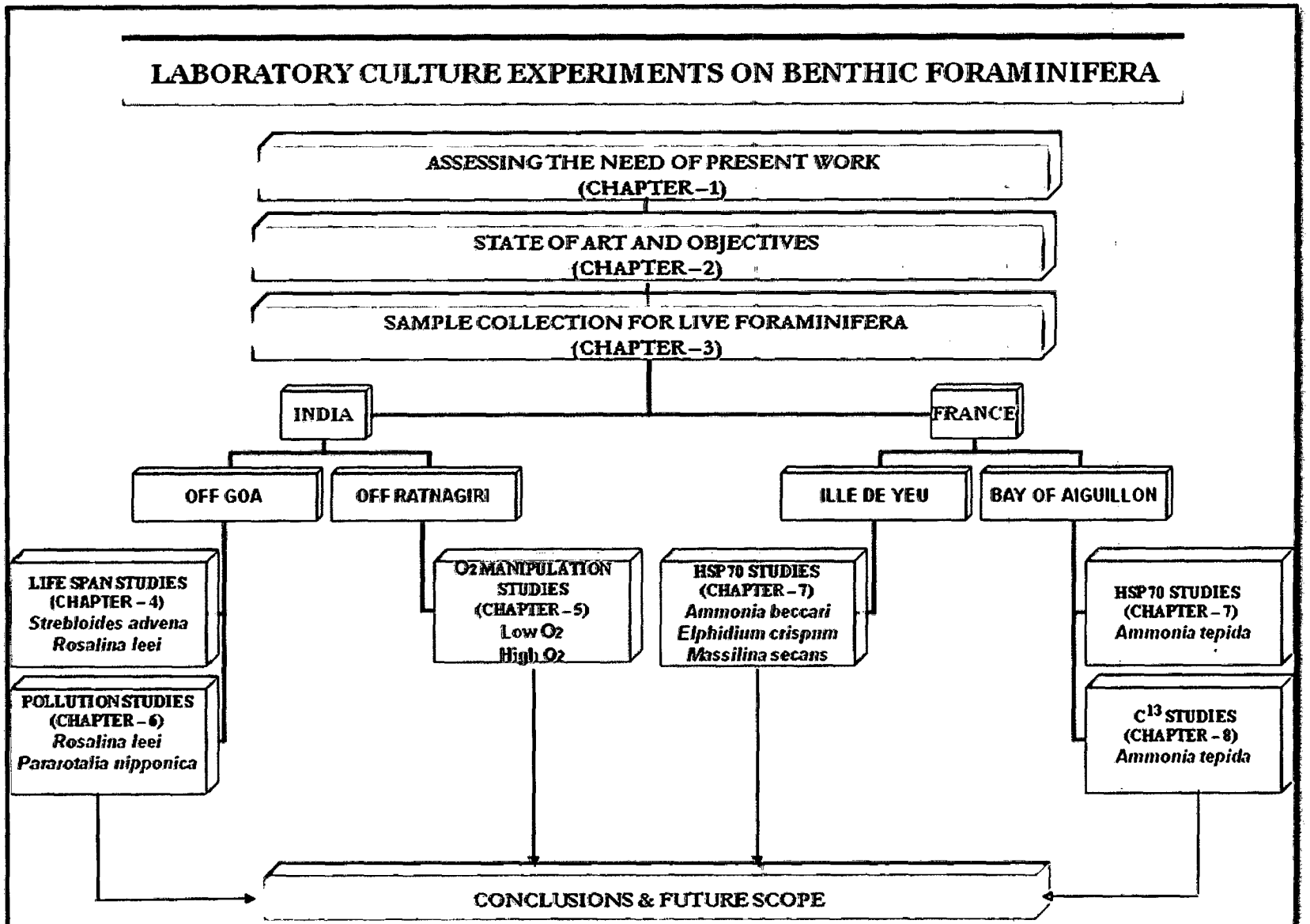


Fig. 3.1: Flow chart illustrating the details of the samples used for various experiments included in the thesis

3.2. Field methods

This is a laboratory based study and the objective was to provide experimental support to various field findings based on benthic foraminifera (as proxies). During the course of this research, samples were collected from different sites and experiments were designed taking in to account the prevailing physico-chemical conditions of each location, the details of which are included in the respective chapters. Samples from following 4 different sites were used for various experiments discussed in this thesis-

- Off Goa, west coast of India
- Off Ratnagiri, west coast of India
- Port Joinville on the Ile d'Yeu, France
- Bay of Aiguillon near La Rochelle, France

The details of the sampling stations and the breakup of the samples studied as a part of this thesis are briefed in table 3.1.

Sampling station	**Geographic location**	**Latitude Longitude**	**Studies based on the sample**
Dias beach	Off Goa, west coast of India	15°27′N 73°48′E	• Lifespan studies (chapter 4) • Experiment to study response of benthic foraminifera to heavy metals cadmium and mercury (chapter 6)
Ratnagiri	Off Ratnagiri, west coast of India	17°30'00''N 72°42'08''E	• Experiment to study response of benthic foraminifera to oxygen manipulations (chapter 5)
Port Joinville	Ile d'Yeu, France	46°43'37''N 2°20'46''W	• Experiment to study the stress protein in a few benthic foraminiferal species (chapter 7)
Bay of Aiguillon	La Rochelle, France	46°17'N 01°10'W	• Experiment to study the stress protein in a few benthic foraminiferal species (chapter 7) • Experiment to study the differential uptake of ^{13}C labeled feed by *Ammonia tepida* (chapter 8)

Table 3.1: Details of the samples used for various experiments of the present work

3.2.1. Off Goa, west coast of India

An ideal area for field collection would be one with close proximity to the laboratory. Dias Beach, Dona Paula, Goa was one such location at stone throw distance from National Institute of Oceanography, Goa off central west coast of India. Situated between 15°27′N latitude and 73°48′E longitudes (Fig.3.2), this rocky beach is protected on either side by rocky cliffs and few rocks can be seen in the sub tidal zones during low tides. The location has two major estuaries namely Zuari and Mandovi draining huge amount of fresh water during southwest monsoon, which leads to large-scale changes in the seawater salinity varying from 11 ‰ to 36 ‰ within short time periods (Rodrigues, 1984). This being the nearest and the dependable source for live foraminiferal specimens, samples were regularly (depending up on the requirement in the lab) collected. Bulk of the study was carried out using samples from this location. The experiments dealing with the growth and reproduction of benthic foraminifera, effect of heavy metals mercury and cadmium were conducted with benthic foraminiferal samples from this location.

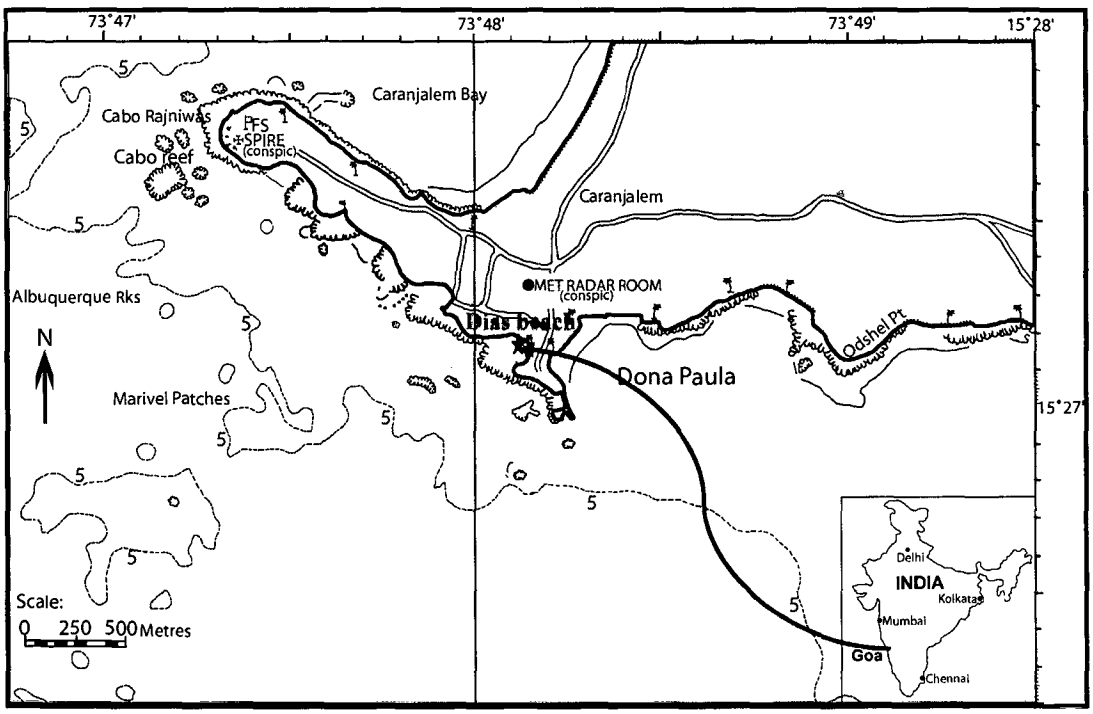


Fig. 3.2 Sampling location: Off Dias beach, Goa, west coast of India

Both sediment and algal (floating as well as the ones attached to rocks) samples were collected in pre-labeled polythene bags containing seawater. Once brought to the shore, the algal samples were transferred to plastic tub filled with filtered seawater

and were shaken vigorously to detach the foraminifera from the attachment, and then sieved over 2 sieves with different mesh sizes, kept one over the other. The sieve kept on the top has a mesh size of 800 μm and is used to get rid of the extraneous material whereas the lower sieve has a mesh size 63 μm that is used to concentrate the foraminifer specimens for the laboratory analyses. At times a third sieve was used below 63 μm size, for specific observation on the finer fractions (Fig. 3.3). The plus 63 μm sample was collected in beakers along with seawater and was brought to the laboratory. The sediment samples were sieved over 63 μm sieve and the filtrate (> 63 μm fraction) was collected in beakers filled seawater and brought to the laboratory. The sea water in the beakers containing the samples (both algal as well as sediment) was filled with almost three times the quantity of the sample. Seawater is also collected from the same area since seawater is used as the media for culturing foraminifers in the lab. Physicochemical parameters like salinity, temperature, D.O and pH of the seawater are recorded by using a hand refractometer, thermometer, iodometry/D.O. meter and pH meter respectively.

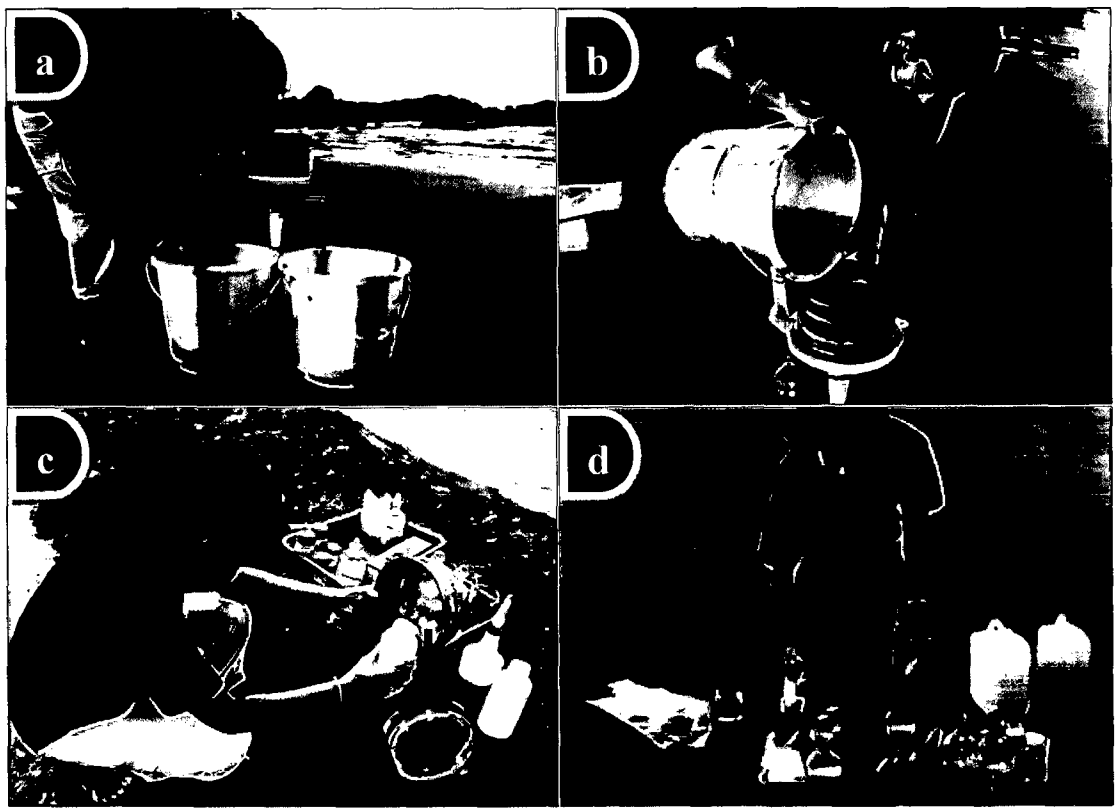


Fig. 3.3: Various steps in initial sorting of algal samples in the field:
a) Algal samples being shaken to detach foraminifera; **b)** Samples being sieved through a set of sieves with different mesh sizes; **c)** Sieved sampled sample being gently transferred from the sieve; **d)** The beaker with the sample and seawater to be transferred to the lab.

3.2.2. Off Ratnagiri, west coast of India

As a part of an Indo-Dutch collaborative program, a box corer specially designed by the Netherlands Institute of Ecology (NIOO) was used to collect 3 box cores at the same location, shelf region off Ratnagiri on the west coast of India (17°30'00''N and 72°42'08''E), at a depth of 50 m onboard ORV Sagar Kanya during cruise SK-211 in the first week of October 2004 (Fig 3.4).

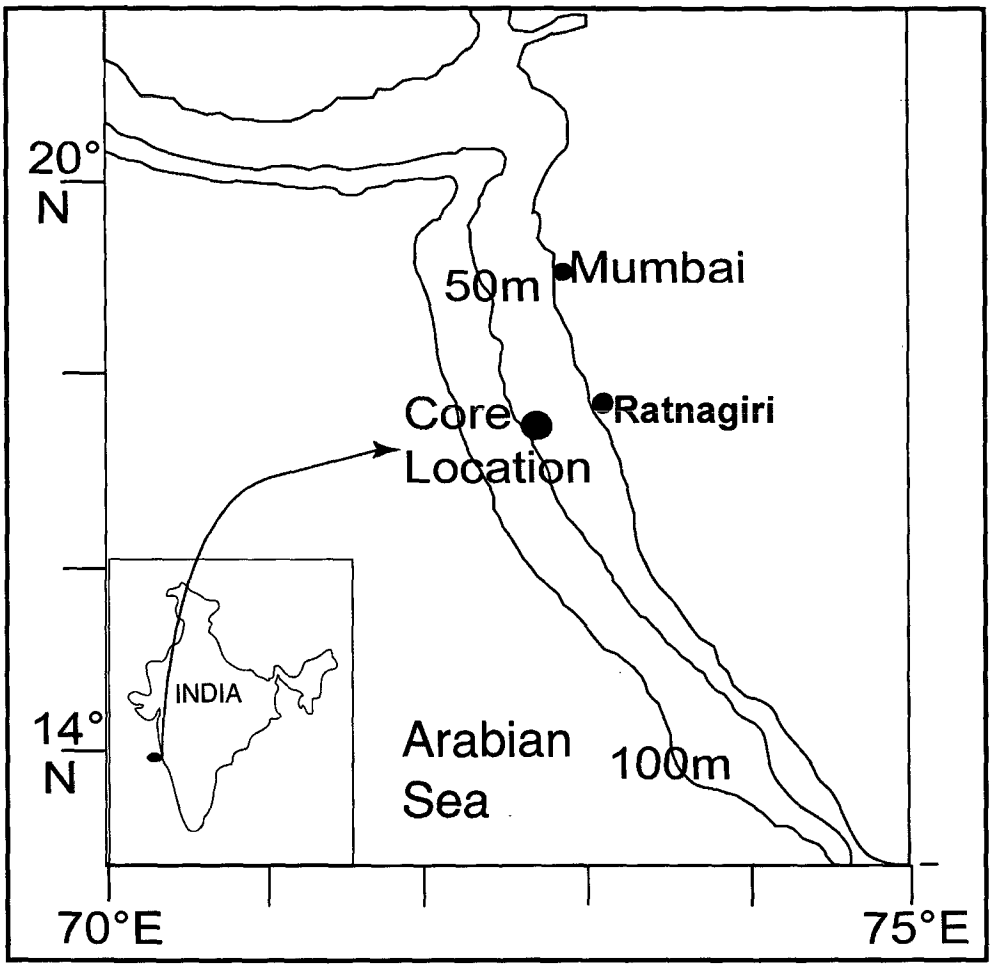


Fig. 3.4: Sampling location: off Ratnagiri, west coast of India

In each of the box cores, plastic liners were inserted carefully so as to collect bottom sediments and the overlying water with their interface intact (Fig. 3.5). These served as experimental cores for the study of foraminiferal response to varying oxygen levels in the water. Bottom water temperature was noted immediately as 11°C. The oxygen concentration in the bottom waters was found to be 68 μmol/l and salinity was measured as 36.15 ‰.

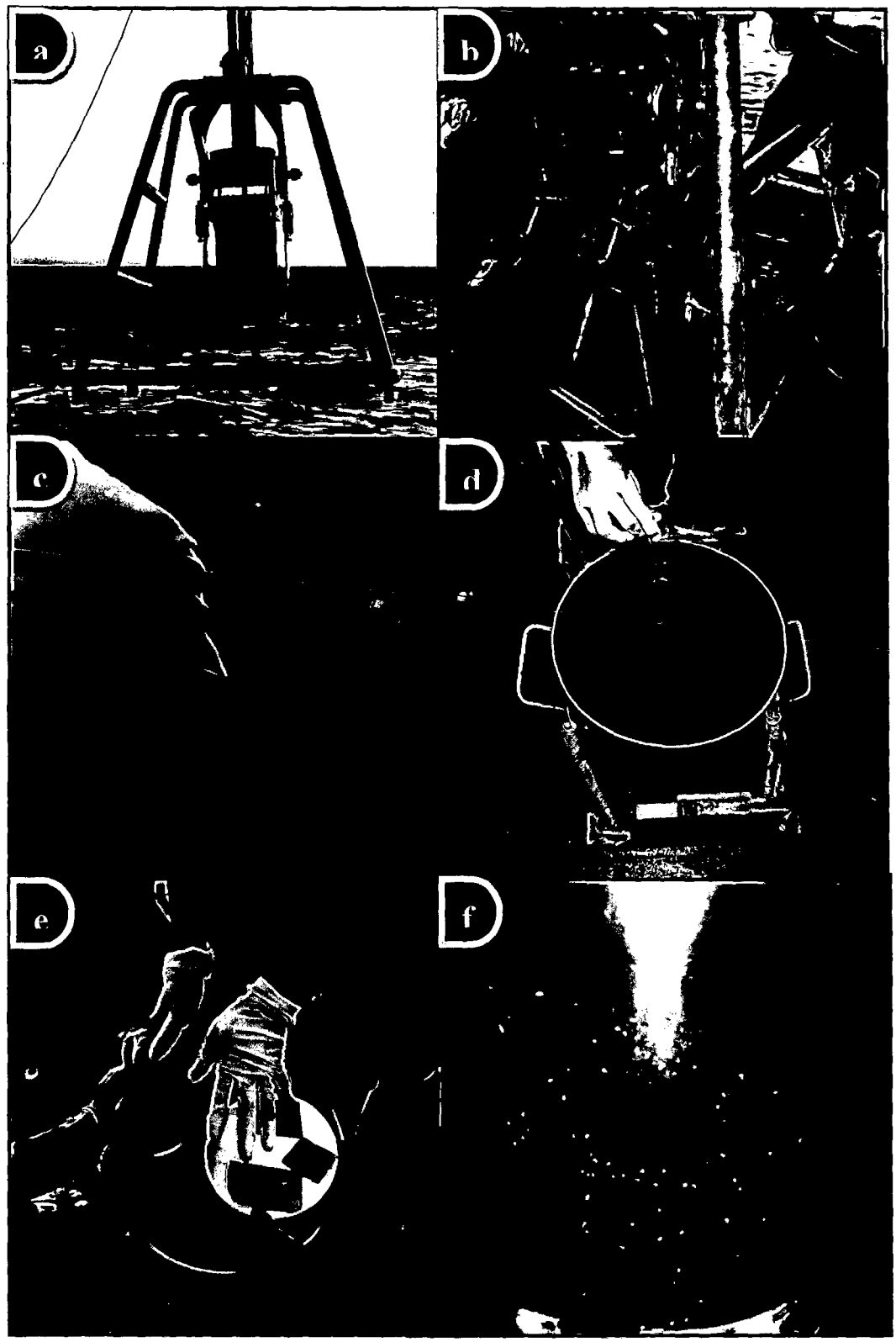


Fig. 3.5: various steps involved in the onboard collection of samples from the boxcore

a) Box corer coming up with the sample; **b)** Dismantling the box core from the corer onboard; **c)** Box core taken out of the corer; **d)** Sediment sample with the overlying water column as collected in the box core; **e)** plastic liners being inserted in to the box core; **f)** Closer look of the plastic liner sample with the intact water column on top and undisturbed sediment on bottom

3.2.3. Ile d'Yeu, & Bay of Aiguillon, France:

Field sediments samples containing *Massillina secans*, *Ammonia beccarii* and *Elphidium crispum* were collected on the beach near the Laboratory of Marine Bio-Indicators (LEBIM) in Port Joinville on the Ile d'Yeu, France , (46°43'37''N and 2°20'46''W) in May 2008. *Ammonia tepida* was collected in the tidal zone in the Bay of Aiguillon near La Rochelle, France, (46°17'N and 01°10'W in June 2008 (Fig. 3.6) and were transported carried to the Micropaleontology lab, University of Tubingen, Germany where the experiments were conducted.

3.2.3.1. Port Joinville on the Ile d'Yeu, France

Ile d'Yeu is located over 20 km off the coast of Vendee, France. Having a NW-SE extension, it is 10 km long and 4 km wide. The NE coast where the harbour is located, is protected from the influence of of the open sea and is characterized by a reduced wave intensity. The mean tidal range of this area is about 4m. Contrary to most other harbors, Port juvenile is not located in an estuarine zone and thereby receives very little freshwater inputs. The salinity temperature variations in this region are 33.6- 35 ‰ and 16-18°C respectively. The range of dissolved oxygen content is reported as 8.3- 10.6 mg/L and pH: 8.04-8.35.

3.2.3.2. Bay of Aiguillon near La Rochelle, France

The Aiguillon Cove (AC) is a large intertidal area (Verger, 1968) of 49 km^2, of which 33 km^2 are constituted of mudflats and 11 km^2 of surrounding salt-marshes. The salt-marshes and the neighboring agricultural areas are drained by a dense network of small channels, which import freshwater in the cove in addition to the Se`vre Niortaise River. The cove is a semi-circular sedimentation basin for silts and clays, which are mainly trapped in its landward parts (Verger, 1968 *in* Leguerriera *et al.*, 2007). It has a gentler bottom slope and a larger mudflat on the southern than on the northern part (1.5:1000 vs. 1.8:1000 and 3.5 vs. 3 km, respectively). The Aiguillon Cove also receives oceanic inputs via the Pertuis Breton.

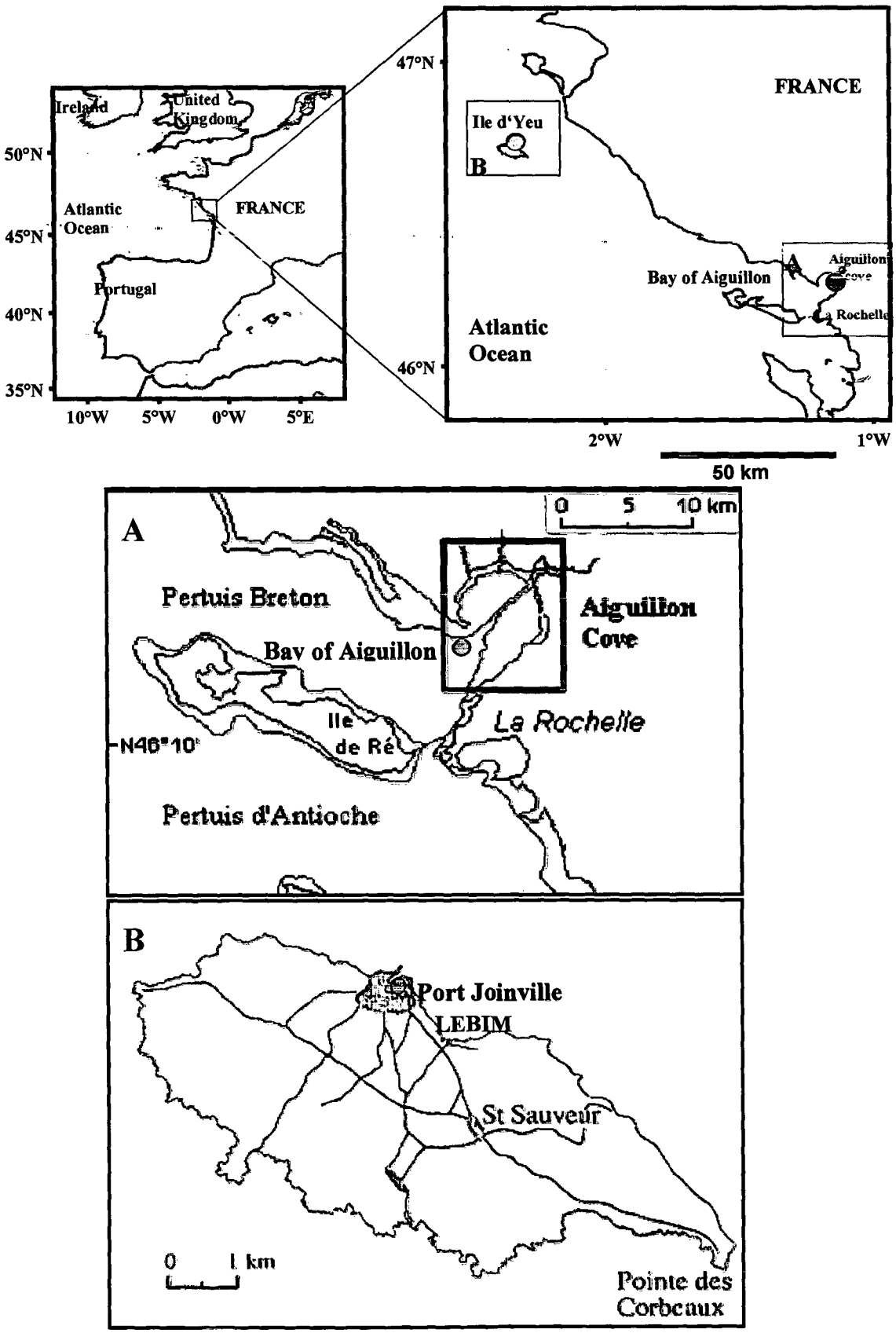


Fig.3.6: Sampling locations: Port Joinville on Ile d'Yeu and Bay of Aiguillon, France

A: Enlarged view of Bay of Aiguillon; **B**: Enlarged view of Port Joinville

The sediment samples were scooped in to the sample containers. Since the sample consists of fine mud, considerable amount of sediment was collected keeping in mind that the working sample will be greater than 63 μm fraction of the sediment. The sample was then gently washed over 63 μm sieve in the field itself in order to get rid of the muddy fraction and to concentrate the foraminiferal samples (Fig. 3.7). It was not possible to wash the samples thoroughly in the field due to rains during the field work. So the preliminary washing of the samples was done in the field and then the samples were properly washed and sieved again later in the laboratory, University of Angers. One more reason sieving the sample in the field was to reduce the volume of the sample which is easy for transportation, as the samples had to be transported all the way from France to the Micropaleontology laboratory in Tubingen, Germany. The samples were washed with the seawater from the same site and the greater than 63 μm fraction remained over the sieve was collected in prelabelled plastic containers along with some amount of seawater, which were then transported to the lab.

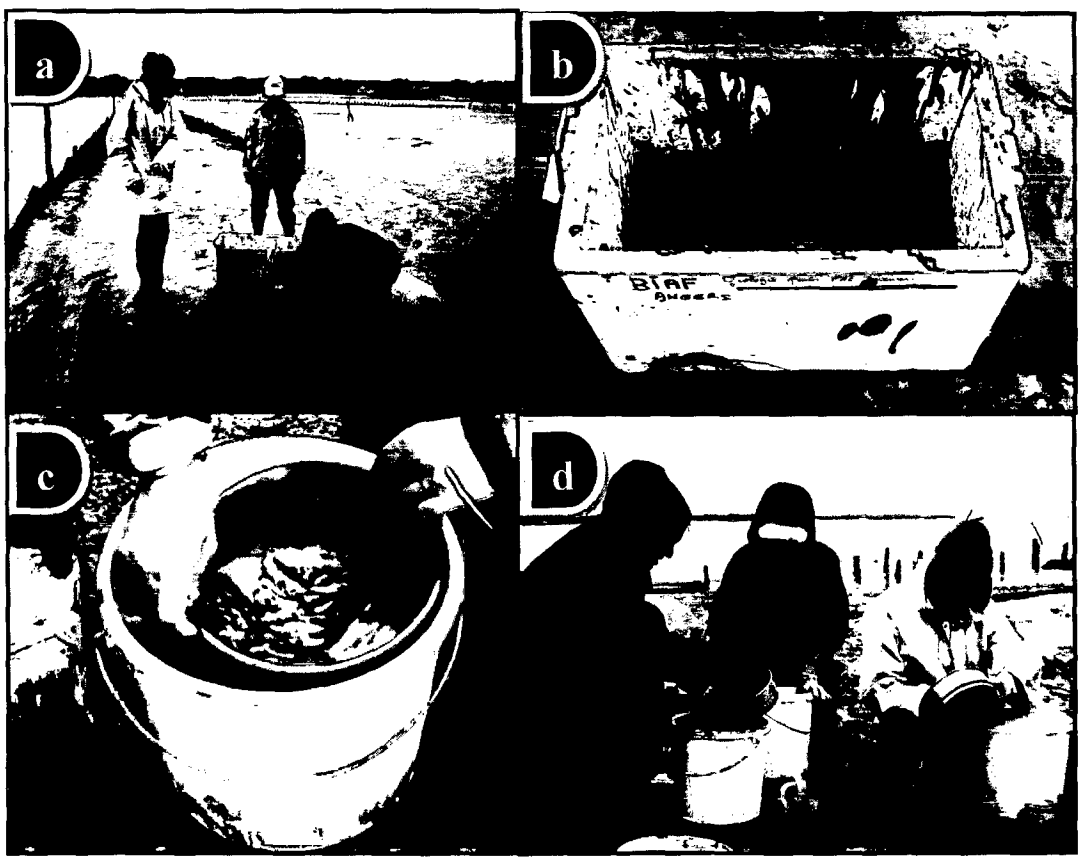


Fig. 3.7: Various steps in initial treatment of sediment samples in the field:
a) Sediment samples being scooped in to sampling box; **b)** Closer look of the collected sample; **c)** Preliminary sieving of the samples in the field; **d)** Sieved sampled getting transferred to prelabelled bottles along with seawater

3.3. Laboratory methods

3.3.1. Laboratory setup

Infrastructure facilities in the Foraminiferal Culture Laboratory (Fig.3.8), National Institute of Oceanography, Goa is comparable to any sophisticated laboratory of its genre. Having the very advantage of its geographical position, proximity to the sea, this laboratory have advantage over many foreign laboratories dealing with this subject, where they have to use artificial seawater transport the live samples from distant places which affect the proper running of laboratory studies that require considerable amount of live samples time to time. Apart from the availability of samples throughout the year (though the peak monsoons prevent collection for security reasons for 2-3 months), this laboratory has wide range of sophisticated instruments and equipments required for the study.

Fig. 3.8: Glimpse of Foraminiferal Culture laboratory, National Institute of Oceanography, Goa, India

Different types of microscopes- stereo zoom transmitted light binocular, trinocular microscopes with high end photographic facilities, inverted microscope with live monitoring facilities and image analyses software (Nikon ACT 2U, Image Pro express) for the effective monitoring of the vital activities and the response of live foraminifera, and for the fine imaging and image analysis; Number of B.O.D. incubators for temperature and light regulations; pH meter, DO meter, refractometer for the effective monitoring of the physicochemical parameters from the field as well as from the experimental system; autoclave, laminar flow for contamination free culturing etc are set for the effective running of the experimental studies on benthic foraminifera.

3.3.2. Storage of samples in the lab

Samples collected from the field were brought to the laboratory and stored in beakers filled with the seawater collected from the same location. Initially, when the sample was fresh, teaming with live organisms and filled with organic matter, the sea water of the beaker containing the samples were changed periodically after approximately every three hours or so to elude the reducing dissolved oxygen levels. After a span of two to three days the periodicity of the water change was reduced to twice a week. The beakers with sample were properly covered in order to prevent evaporation of water and a consequent rise of salinity. Periodic or sporadic aeration of the sample was a requisite.

3.3.3. Distinguishing live from dead foraminifera

It is important to distinguish living from dead individuals and to have the general idea about the relative proportion of the two in the samples collected for culture studies so that attention and effort can be paid to the more promising ones in order to establish healthy cultures in the laboratory.

A wide variety of methods of distinguishing living from dead foraminifera have been in use for decades. In an excellent review, Bernhard (2000) explained the non-terminal (without killing the specimens) and terminal methods (in which specimens are killed) and discussed their advantages and disadvantages. The choice of technique depends on the objective of the study. Though the present study mostly deals with the non-terminal methods of identifying live foraminifers, terminal method (Rose Bengal) was adapted for one of the field based study (onboard experiment) dealing with the

foraminiferal responses to varying oxygen levels and hence discusses the basics of both techniques in brief in the coming sections.

3.3.3.1. Terminal methods

In Terminal method of identifying live foraminiferal specimens, organisms are killed in order to determine their live status. Among the terminal methods, rose Bengal (Walton, 1952) method is most widely used, though a number of non-vital stains like eosin (Rhumbler, 1935) and Sudan Black B (Walker *et al.*, 1974) were applied for the same purpose by various workers. Rose Bengal is a protein stain, first used as a means to distinguish living foraminifera from dead, by Walton (1952). It adheres to the protein, imparting a magenta colouration to the specimen. It is inexpensive and comparatively easy to use as no sophisticated instrumentation is needed. So it is ideal while studying large number of samples especially during abundance studies. The chemical formula for rose Bengal is $C_{20}H_2Cl_4I_4Na_2O_5$.

Rose Bengal (rB) staining is the most appropriate for analysis of large number and bulk samples, which many times need to be preserved. But this method is so often discussed these days for its disadvantages. Off late, number of workers reported that rose Bengal being protein specific, adheres to the dead as well as living cytoplasm and stains dead protoplasm for prolonged durations even after the death of the individuals. This causes an erroneous over estimation of standing stocks (Bernhard, 1988; Hannah & Rogerson, 1997). As this method was used in the experiment to study the response of benthic foraminifera to oxygen manipulations (Chapter 4), we conducted a small experiment to observe the effectiveness of this technique and the observations are described below.

We stained both live and dead (killed for the experiment) specimens in the laboratory, in an effort to verify the effectiveness of the rose bengal staining method as it is the most common method we apply when studying bulk samples from field. Our observations are in general agreement with the findings of Bernhard (1988) and could see that rose bengal is capable of staining both the living as well as dead protoplasm. However closer observation of the stained specimens revealed a difference in the staining pattern between living (living at the time of staining) and dead specimens. The living specimens had a tendency to acquire deep denser stain up to one additional chamber, than those with visible protoplasm (Fig.3.9).

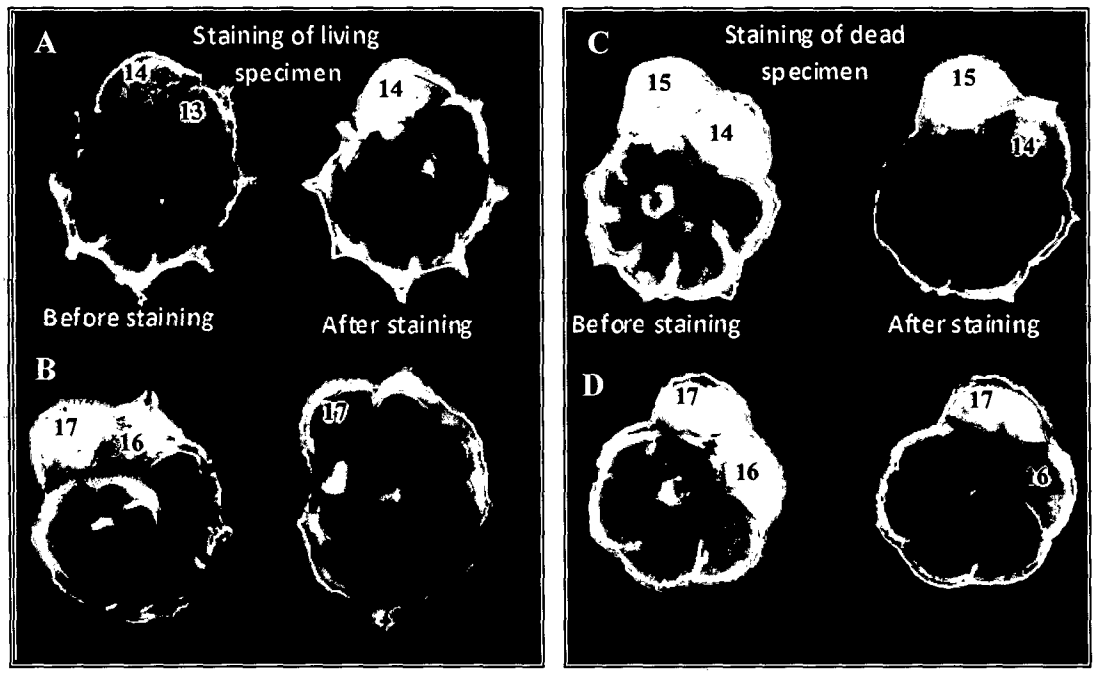


Fig. 3.9: The difference in the pattern of staining between living (at the time of staining) and dead (killed for the experiment) specimens of *P.nipponica*

For example, if we compare the live and dead stained specimens in Fig.3.9, we can see that- the live specimens A and B show visible protoplasm, in 12 and 15 chambers and after staining, the same specimens are densely stained to up to 13 and 16 chambers respectively. Whereas in the dead specimens, visible protoplasm is seen up to 13 chambers in specimen C and 15 chambers in specimen D, but no additional chamber is seen stained in either of the specimens other than the slight pink coloration of the superficial staining.

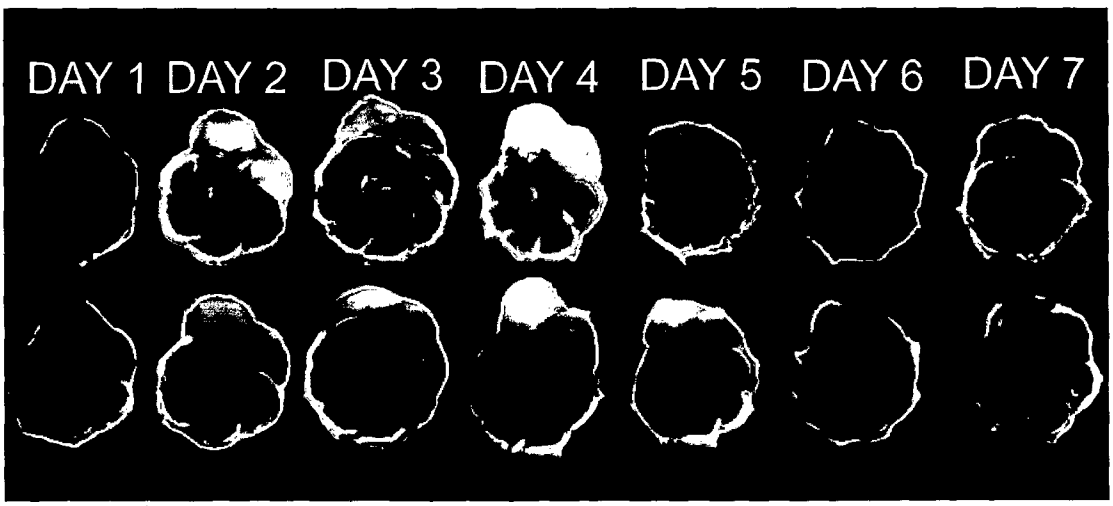


Fig. 3.10: The staining of *Pararotalia nipponica* specimens from 1-7days after their death

In line with the findings of Hannah & Rogerson, (1997), we observed that the foraminiferal specimens were getting stained even seven days after their death and there was no significant change in the pattern of staining with in this one week period (Fig.3.10). For some reasons, the experiment could not be extended for more than seven days time, but we hope this observation itself is sufficient to support that the rosebengal staining has to be applied very cautiously, lest it will lead to erroneous over estimation of standing stocks. However none of the alternative staining techniques could fully substitute this method due to its simplicity in usage and application to large number of field samples which needs preservation after staining. Techniques like cell tracker green which is supposed to be most practical among other sophisticated molecular based techniques are still not handy when it comes to field as it requires incubation before fixing the stain. Taking this scenario in to consideration, rose bengal is still to be substituted with a better staining technique. If used under caution by experienced hands, this method can produce good results.

The practical way of reducing error is to understand the pattern of staining thoroughly and try to ascertain the live status of the specimen. Though it is advisable to use this method in conjunction with other staining methods it is not always practical when dealing with huge number of field samples.

3.3.3.2. Non terminal methods

Non terminal methods are those which determine whether the organism is alive without killing it. This method requires the specimens to be maintained at suitable ambient environment conditions in order to ensure their living conditions. For experiments with living benthic foraminifers, it is clearly necessary to use non-terminal techniques, which in no way harm the organism or interfere with its normal activity. The following non-terminal methods were applied to distinguish the living specimens.

- **Sheen/luster of the test:** Healthy specimens characteristically possess a lustrous test from which light is brilliantly reflected than those of the dead specimen. Though this is invariably true in case of all living benthic foraminifers, the specimens soon after their death also hold this luster for quite some time. So this is rather a preliminary aid to be used cautiously with other factors.

- **Natural colour:** Empty tests have a different appearance than those containing protoplasm (Fig. 3.11). Protoplasm renders peculiar coloration to the foraminiferal tests. This serves as a very useful feature to identify the live foraminifers from the dead ones. In case of test with protoplasm, the most frequent colors encountered in most benthic foraminifera are green and brown and all intermediate shades. The colors are not only due to the actual color of the protoplasm but also due to the color of the symbiotic algae living in the test or algae captured for food. Though this is a very useful criterion that helps to identify the live organism, it is not to be used alone, and should be supplemented by other confirmation.

- **Apertural bolus:** A mass of food/detritus is normally seen in the vicinity of the apertural openings of the living individual which is usually indicative of an actively feeding individual (Fig. 3.11). Though it is most commonly seen around aperture, at times some foraminifera collect food material around its body to form a cyst, usually this happened prior to asexual reproduction in many species and is known as "reproductive cysts". It is reasonably safe to conclude that the appearance of food material around the apertures (previously devoid of any) indicates viability, it should not be the only factor for such decision, since these masses persist at times even after death of the organism due to some unfavorable causes or due to reproduction.

- **Cytoplasmic streaming/ pseudopodial activity:** this is in a way the best method to confirm the living status of a foraminiferal specimen. But it is to be noted that the specimen may not be extending pseudopodia every time under observation. On very slight disturbance also they retract the pseudopodia, and again will extend the pseudopodia under favorable conditions. Most of the times, careful and continuous monitoring is required in order to capture the pseudopodial activity from foraminiferal specimens. The length of pseudopodia varies in different species and at times it was seen that the length of the pseudopodia reach several times the size of the test. Not only the mature specimens extend pseudopodia, even the new born juveniles show strong pseudopodial activity like movement, food capture and most importantly the chamber formation. Prior to the addition of new chamber, the pseudopdial reticulum gathers food particles and forms an outline of the to-be formed chamber, then the protoplasm is filled in to it to complete the process.

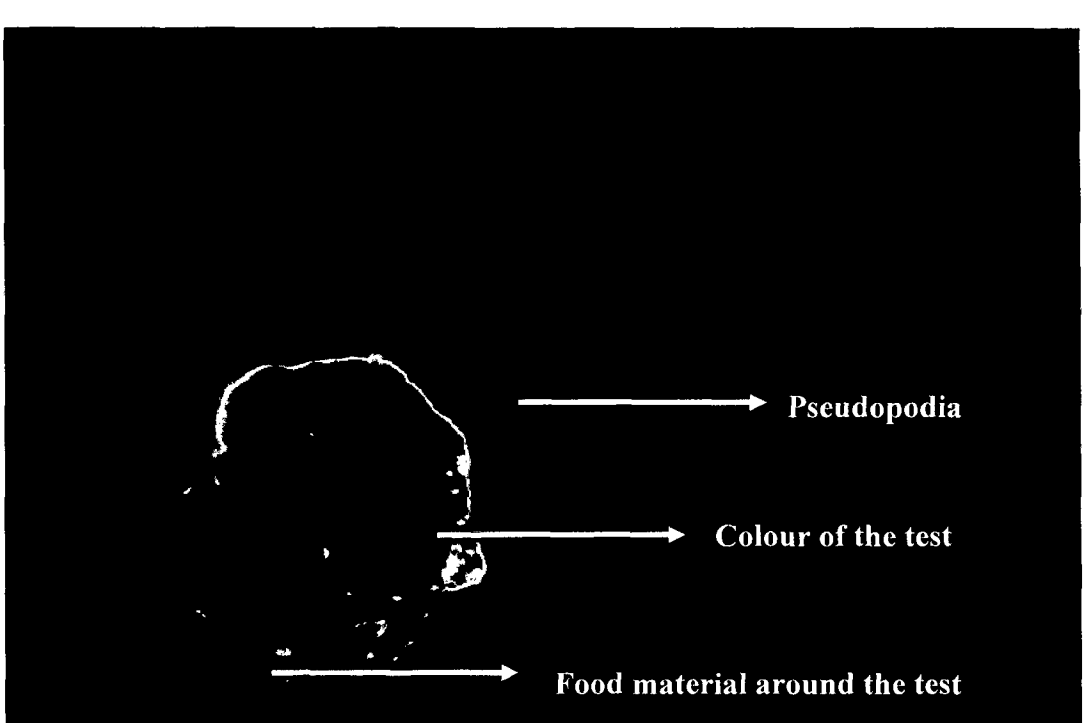


Fig. 3.11: The appearance of a healthy living benthic foraminiferal specimen (*Cymbaloporetta plana*) from the laboratory culture

- **Positive phototaxis/ negative geotaxis:** In the laboratory, we have noticed several specimens crawling up the walls of the storage vessels/ culture dishes. In this case there is no further confirmation required regarding the live status of the specimen; rather it is indicating that the specimens are in a very healthy condition. But this method cannot be applied when sorting large number of specimens for experiment.

  In order to distinguish the living specimen the above mentioned non-terminal methods are used in conjunction with each other as per the response of the specimens.

3.3.4. Maintenance of live foraminifera:

The live foraminifers were identified and were separated with the help of a fine paint brush/ pipette controller and kept in multiwell culture dishes containing seawater of known salinity. They were maintained in different set of salinity-temperature conditions in various culture dishes. The culture dishes were maintained at different

temperatures in B.O.D. incubators. Some dishes were also maintained at room temperature.

Seawater of different salinities was prepared by adding ultra pure deionised distilled water (lower salinities) and by evaporating at 40-45^{0}C (higher salinities). The salinity of seawater was determined using a hand refractometer. Thus by keeping the foraminifers under observation, the salinity and temperature optima for growth and reproduction of each species were determined. The dishes were periodically checked for the salinity level, the health and well being of the foraminifers.

The healthy foraminifers thus maintained in the laboratory and the offsprings, which are reproduced under the laboratory conditions, are used for further experiments (Chapters 4,6,7,8). The set-up for each experiment vary widely, the details of which are explained in respective chapters to properly convey the idea.

CHAPTER 4

GROWTH AND REPRODUCTION IN BENTHIC FORAMINIFERA

Chapter 4

Growth and reproduction in benthic foraminifera

"The moment of enlightenment is when a person's dreams of possibilities become images of probabilities."

- Vic Braden

4.1. Introduction

Boltovskoy & Wright (1976) while listing the "unresolved problems" in their classical book "Recent foraminifera" stated "one of the urgent tasks in the future study of foraminifera is the investigation of living specimens, their reproductive cycles, their physiology, ontogeny and ecology. These studies must be conducted in laboratory cultures..." Even after one and a half decade, Lee & Anderson (1991) while writing introduction to book on foraminifera expressed their opinion that "....it has become clear to us, from our perspective as editors, that we really know very little about most aspects of foraminiferal biology and much further research is required on the life cycles of foraminifera to provide background information essential for classical and molecular genetic research." Status has not changed much since then. The present chapter is designated to the laboratory studies on the growth and development of benthic foraminifera under laboratory conditions with special emphasis on *Strebloides advena* and *Rosalina leei*. The very basics of the foraminiferal biology and ecology are discussed in brief in the beginning of the chapter in order to make the terminologies and parameters used in the whole thesis/entire work on culturing understood.

4.2. The living organism

Although foraminifera are often thought of as simply being amoebas possessing an outer shell, there is more to them than that. The shell is commonly known as 'test' (hard part) which act as support and protection for the majority of the protoplasm that constitute the 'living' (soft part) part of foraminifera. The protoplasm of foraminifera contains all the organelles and inclusions typical of other animal cells as well as some of which are unique to foraminifera (Boltovskoy & Wright, 1976). The protoplasm renders a typical colour to the living organism (Fig. 4.1), which helps primarily distinguish the living from the dead foraminifera. If the wall is transparent the most frequent colours encountered are green, yellow, orange, brown and all intermediate shades.

This colour is due to the combined effect of protoplasm and symbiotic algae or the algae trapped for food. Colour variation may be caused due to nutrition variation and symbionts.

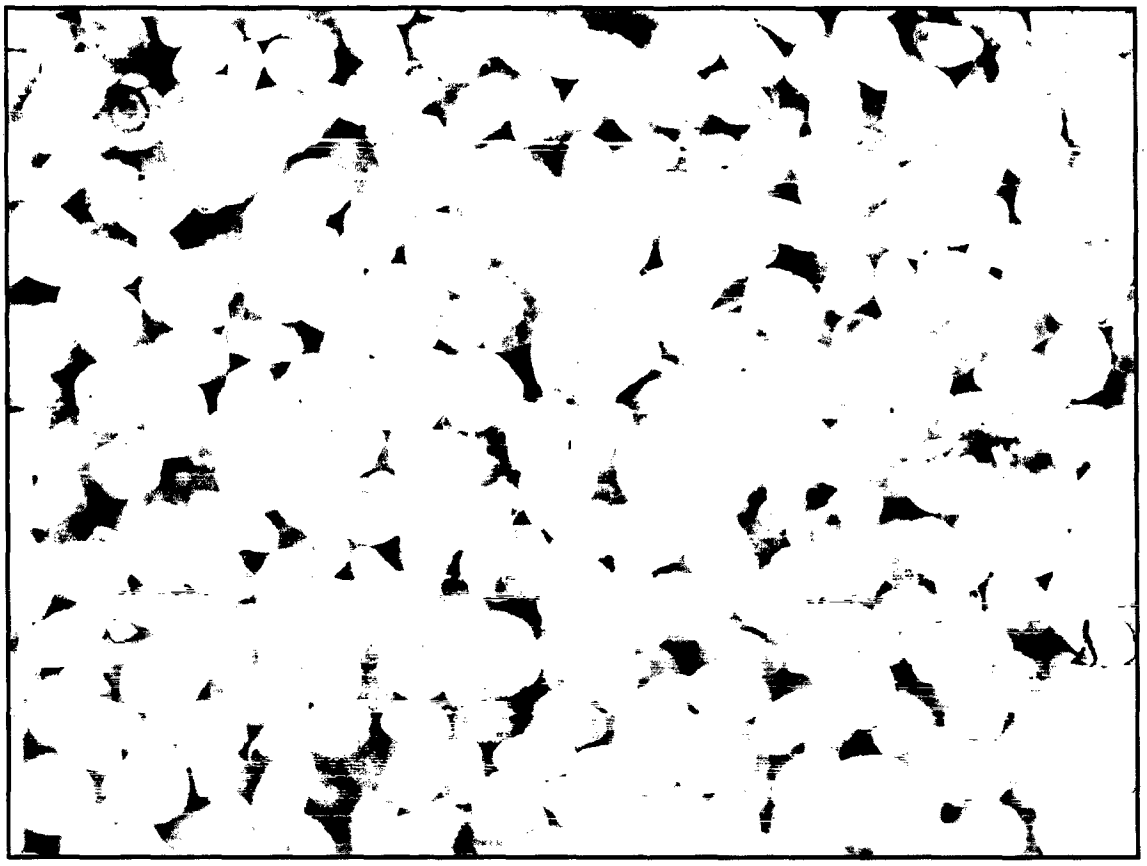


Fig. 4.1: A bunch of living specimens of *Ammonia tepida* illustrating the typical protoplasmic coloration

In multi chambered forms, the protoplasm can be differentiated in to a vegetative segment, which contains the food particles and food residues and comprises the last chambers and a chromatic segment, which contain the nucleus and other cell inclusions and occupy the initial chambers. The foraminiferal protoplasm can be divided in to internal protoplasm (endoplasm) occurring within the test and external protoplasm (ectoplasm) located outside the test in contact with the environment. The endoplasm is responsible for the general metabolic processes of the organism whereas the ectoplasm is intimately involved in the secretion of the test and most of the interchange between the organism and the environment occurs across it. It has the ability to simultaneously dissolve the calcareous material on its external surface and secrete calcareous test on its inner layer (Banner, 1971). The ectoplasm forms elongate pseudopodia, which give the organism the ability to move and attach itself. The length attained by the pseudopodia is variable.

These cytoplasmic threads, the thinnest of which are <1 μm across, may reach a distance of several times to their test diameter. If a foraminifer is disturbed, the pseudopodia retract but re-emitted from the test within a relatively short time.

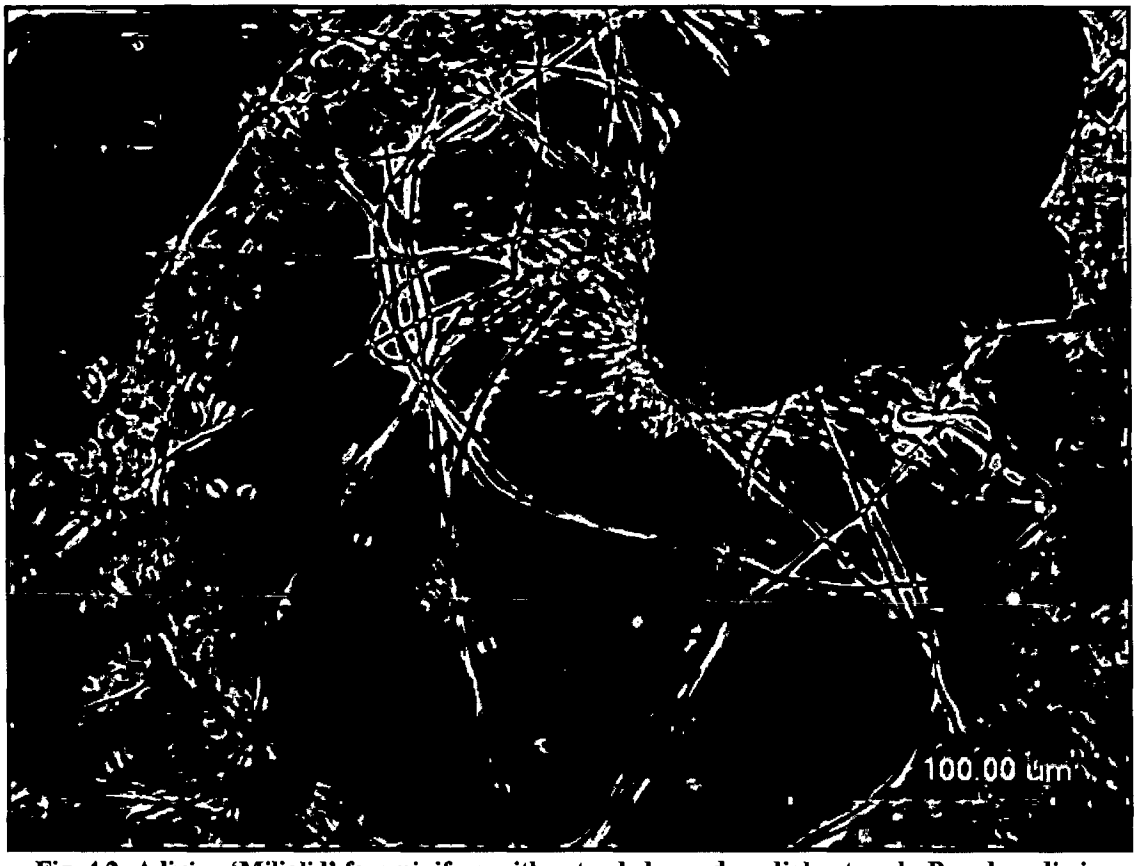


Fig. 4.2: A living 'Miliolid' foraminifera with extended pseudopodial network: Pseudopodia is visibly emitted from the apertural opening only

The imperforate foraminifera extend their pseudopodia from their aperture (Fig. 4.2). In the perforate forms, aperture is the primary orifice through which pseudopodia come out and pores may also serve this function (Fig. 4.3). The type of pseudopodia in foraminifera is not only branching, but the branches might also fuse. Such connections between branches are called anastomoses. This branching pattern creates a pseudopodial network. Granules occur within the pseudopodia and are transported along them. They can simultaneously move outwards and inwards in the same pseudopodium. The pseudopodia looks like a much branched network with numerous small granules, and is called granuloreticulopodia.

The pseudopodia not only provide surface for respiration, but also perform functions like feeding, locomotion, test building, metabolite release, adhering etc. The pseudopodia act as sensory devices warning the organism of nearby objects and changes in the chemical environment.

When comes in contact with food particles, the pseudopodia surrounds it and transport it to the ectoplasm. Within the pseudopod there are 2 currents simultaneously moving towards and away from the test (Boltovskoy & Wright, 1976).

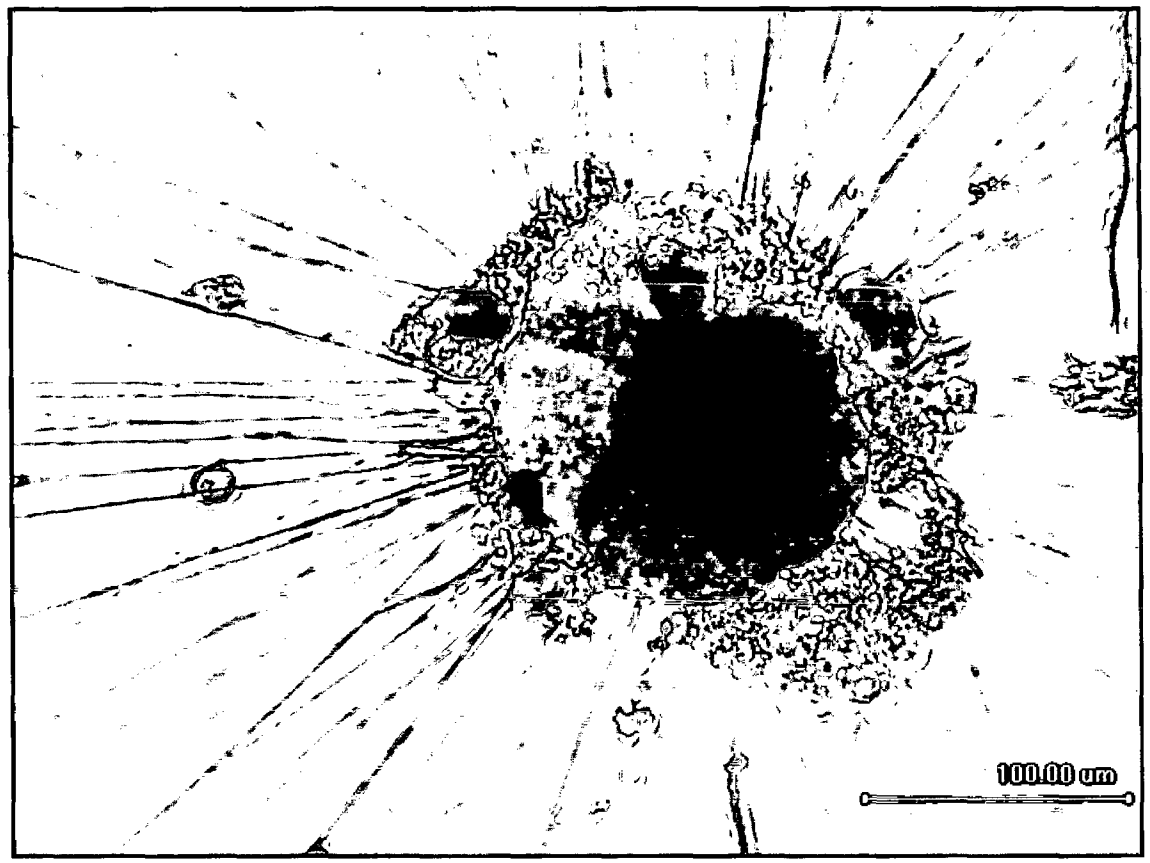


Fig. 4.3: A living foraminifera '*Trochamina*' with extended pseudopodial network: Pseudopodia is visibly emitted from all the pores

These currents carry the nutritive material to the endoplasmic layer and then in to the test either through the aperture or the other test perforations. The digestion of the nutrient particles may occur even as they travel toward the final assimilation in the cytoplasm. The indigested food particles are carried by the cytoplasmic currents and expelled from the test.

4.2.1. The test

The soft part of living foraminifera is enclosed within an external covering commonly referred to as test. The foraminifers differ from many other exoskeletal invertebrates (Boltovskoy and Wright, 1976) in that the soft parts of the organisms sometimes extend beyond the exoskeleton i.e. the test. The test is composed of one or more chambers. When more than one chamber is present the initial (first formed) one is generally the

smallest and is known as the proloculus. The chambers are separated by septa whose intersection with the test wall produces a line of contact or suture and the adjacent chambers are interconnected by an opening called the foramen.

Hence the organisms are named "Foraminifera" meaning "foramen-bearing". The primary orifice through which the protoplasm enters or leaves the test is known as aperture. The morphological characters of the test are used to distinguish one species from another. The three different views of a foraminiferal test with the main morphological features are given below (Fig. 4.4).

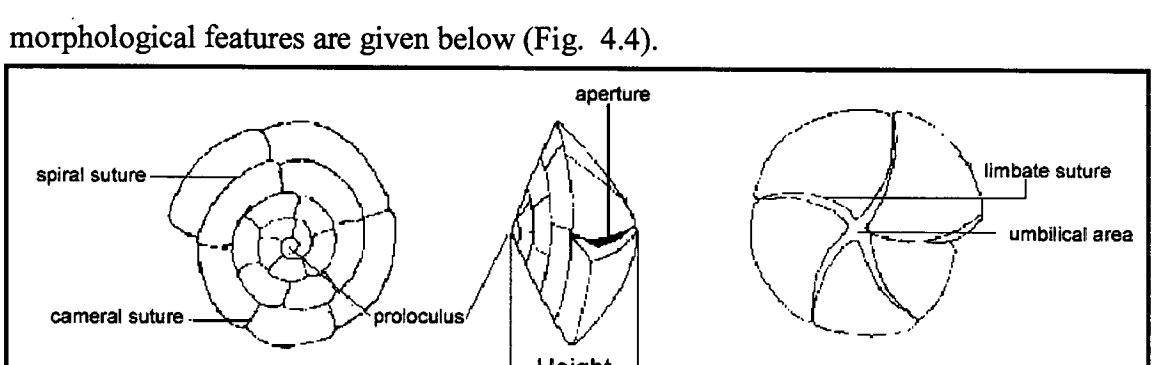


Fig. 4.4: Three possible views of a rounded foraminiferal test

4.2.2. Composition of the test

Composition and construction of test walls are of great systematic importance in foraminifera. There are 4 distinctive types of walls:

- Chitinous: These walls are the most primitive. Fossil examples are not well known due to the ease with which erosive and transportive agents destroy this delicate material. It is actually not true chitin but a tectinous substance (combination of protein and various hydrates of carbon) with different characteristics. These walls are usually transparent and lack pores.
- Agglutinated: They are commonly composed of a layer of chitinous material capped by cemented detrital sediments. Both the cementing agent and detrital material constituting the agglutinated tests are highly variable. The agglutinating grains usually include quartz, mica plates, coal, volcanic glass, rutile, ilmenite, feldspar, tourmaline, zircon, amphibole, sponge spicules, diatoms, mollusk fragments, cocoliths and even foraminiferal fragments, which form a tightly interlocking mosaic with spaces between them filled with finer grains and cement.

- Calcareous: Both calcite (hexagonal) and aragonite (orthorhombic) forms of calcium carbonate is used for construction of calcareous walls. Whether a foraminifera will posses calcite or aragonite wall will entirely depend on the phylogenetic origin of the species rather than ecology.

4.2.3 Factors affecting the life process of benthic foraminifera:

A large number of interrelated factors influence the vital activity of foraminifera and their distribution; a brief description is given below.

- Temperature: This is the primary factor which controls the geographic distribution of species, and is an important factor affecting vertical distribution. The range of temperature fluctuation is probably as important as the absolute temperature. It has a great deal of control over the vital activities of foraminifera. Each species has certain tolerance limits as well as a temperature optimum. A species can only tolerate temperature extremes for a short period. The sea water temperature influences the test size and morphology of the test. Although the majority of observations indicate that increased size is a result of lowered temperature, this relationship is not perfectly understood.
- Salinity: The sea water salinity also affects the geographic distribution of foraminifera. Because of the relatively uniform distribution of salinity in most of the open ocean, this effect is much more pronounced in areas where there is considerable mixing of fresh water (coastal areas). Not only the amount of dissolved salts but the daily and seasonal variation in salinity affects the distribution and occurrence of foraminifera. It is directly related to the vital activity of foraminifera. Each species has certain critical limits as well as an optimum salinity for various physiological functions. Salinity can affect test morphology, primarily size and ornamentation. Most observations indicate a reduction in size and loss of ornamentation near the salinity tolerance limits.
- Depth: Water depth is an important ecological factor, but its isolated effects are not well understood. It may influence foraminifera only because of depth related changes that occur in other environmental parameters; e.g. as depth increases the pressure increases, the temperature decreases, and carbonate solubility increases. Water depth-related changes have a greater effect on the vertical changes of calcareous species than on those of agglutinated species. Depth can affect the morphology in variety of ways; there is a tendency for many species to become (a)

more rounded with an increase in depth, (b) to increase distribution their ornamentation in deeper water (although many do not) and (c) marked changes in size with an increase in depth.

- Nutrition: Nutrition is among the most important factors governing foraminiferal distribution and abundance. The physico-chemical ecologic factors (temperature, salinity, depth, turbidity, illumination, etc) set the gross limiting conditions for distribution, but within these limits it is often difficult to effect correlations between these factors with quantitative distribution of foraminifera. The nutritional requirements, while not well understood, probably determine the finer distributional patterns. It may influence the size and morphology of the tests. Reduced nutrition tends to result in undersized specimens whose chambers are less regular in shape.
- Substrate: The substrate seems to have some effect on the foraminifera; there are certain species which are found consistently associated with certain type of substrate. Although the correlations are not very good, the bulk of the observations suggest that fine sand mixed with some shelly fragments and silt or clay support the richest standing crop of benthonic foraminifera. The sedentary forms exhibit the greatest morphological variation as a result of substrate condition. The organic content of the substrate can provide nutritive matter and thus be beneficial to foraminiferal development, but excess of it can result in increased acidity and be detrimental to foraminiferal development.
- pH: Low pH creates a stress situation in which calcareous specimens must spend considerable energy recalcifying their tests and is detrimental to the foraminiferal organism and thus restricts its distribution. Low pH values in the substrate may also cause the dissolution of empty tests. Little is known about the effects of highly alkaline (high pH) conditions.
- Trace Elements: Some trace elements in very specific amounts may be essential to the existence of foraminifera like other marine organisms. In excess amounts these elements as well as the unwanted elements can cause morphological variants and dwarf faunas.
- Oxygen concentration: It has been reported that low oxygen concentration can reduce the number of species, enhances the abundant development of certain species and also can at times alter the morphology of specimens. Though this is a factor which can affect the foraminiferal populations, it is not a limiting factor for their existence (Boltovskoy & Wright, 1976).

4.3. Life cycle and life span of benthic foraminifera

Both these terms 'life cycle' and 'life span' sounds similar and are quite often used interchangeably. But it is very much relevant here to specify the difference between the two as with progression, this chapter deals with both. Life cycle is the series of changes in the growth and development of an organism from its beginning as an independent life form to its mature state in which offspring are produced or simply it is the entire sequence of developmental stages of an organism. A life cycle is not a time measure - it describes the progress of the organism through its different stages whereas life span is a time measure - the interval between the birth and death of an organism.

4.3.1. Life cycle studies on benthic foraminifera

In simple organisms, such as bacteria, the life cycle begins when an organism is produced by fission and ends when that organism in turn divides into two new ones. In organisms that reproduce sexually, the life cycle may be thought of as beginning with the fusion of reproductive cells to form a new organism. The cycle ends when that organism produces its own reproductive cells, which then begin the cycle again by undergoing fusion with other reproductive cells. The life cycles of plants, algae, and many protists often involve an alternation between a generation of organisms that reproduces sexually and another that reproduces asexually (www.crsep.org).

The benthic foraminiferal life cycle coincides with the reproductive cycle as reproduction usually terminates the life of the parent specimen in most of the cases leaving some exceptions. The classical life cycle of foraminifera displays an alternation of generations (Fig. 4.5). Gamogamy and agamogamy are the two periods in the life cycle of foraminifera representing the sexually reproductive and asexually reproductive stages respectively. The sexual generation (gamont) alternates with the asexual generation (schizont). The microspheric form with a small proloculus and large test is produced by the union of zoospores; a kind of sexual reproduction and the megalospheric forms with large proloculus and small test is produced by budding.

The number of nuclei in the foraminifera is generally variable and depends on the reproductive generation. The individuals that reproduce asexually (megalospheric forms) posses a large number of nuclei while those that reproduce sexually (microspheric forms) have only one nucleus. The micro and megalospheric forms of the

same species are so different that there are instances where they have been assigned to different genera or species. This morphological difference is called dimorphism.

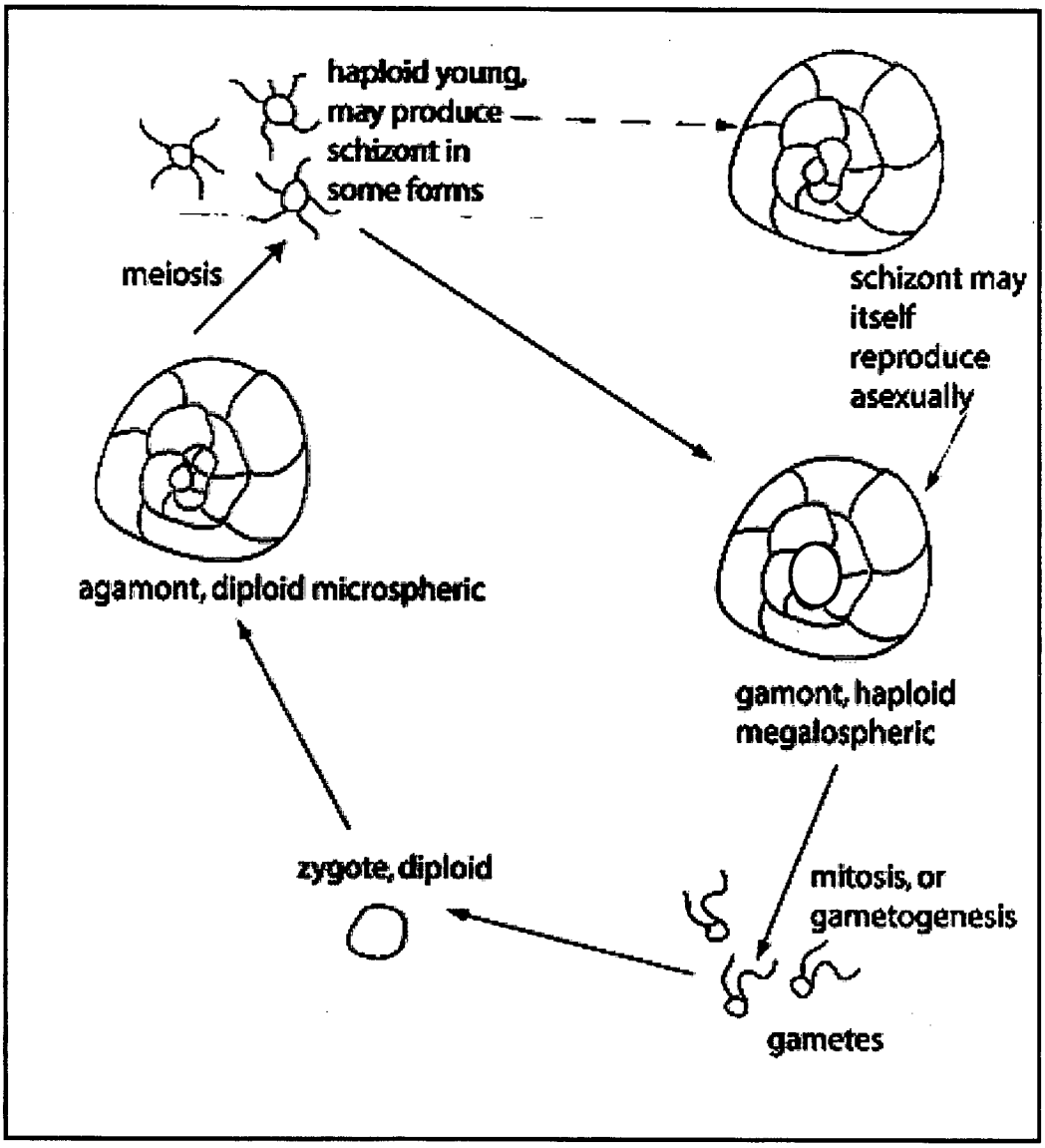


Fig. 4.5: The classic life cycle of benthic foraminifera with alternating sexual and asexual generations (after Goldstein, 1999)

As a general rule, the number of microspheric specimens is considerably less than the number of megalospheric forms. But the opposite situation prevails when the specimens live under unfavorable conditions. Reproduction, like any other physiological trait is species specific in foraminifera. As of date, reasonably complete life cycles are known for fewer than 30 of the 40,000 extant species of foraminifera. In spite of this limitation, these relatively few well documented life cycles illustrate a significant range of variation in the life cycle and in the corresponding morphological variation imparted to the test. Table 4.1 summarizes the general information on the life cycles of various foraminiferal species by the previous workers. Though this table is not covering the

entire information on lifecycle studies, it contains a major amount of information in this regard.

Species	Alteration of generations	Morphology	References
Allogromia laticollaris	facultative budding, plasmotomy	Variable	Arnold 1954b; McEnery & Lee 1976
Heterotheca lobata	obligatory	gamonts generally larger than agamonts	Grell, 1988
Myxotheca arenilega	? obligatory	agamont larger than gamont	Grell, 1958c
Iridia lucida	? obligatory	no morphological difference between gamont and agamont	LeCalvez, 1936a, 1938
Nemogullmia longevariabilis	unknown	no morphological difference between gamont and agamont	Nyholm 1956
Saccammina alba	facultative; budding, plasmotomy	gamonts larger than agamonts	Goldstein 1988
Ovammina opaca	facultative; budding,	secondary pores form in circumapertural ring in test of gamont prior to release of gametes	Dahlgren, 1962, 1964; Goldstein, unpublished observations
Cribrothalammin a alba	facultative; budding,	secondary pores form in test of gamont prior to release of gametes	Goldstein & Barker 1988, 1990; Goldstein, unpublished observations
Patellina corrugate	obligatory	reversed test dimorphism	Myers, 1935a; Grell 1958c
Spirillina vivipara	obligatory	reversed test dimorphism	Myers, 1936
Spiroloculina hyaline	apogamic	not applicable	Arnold, 1964
Fissurina marginata	apogamic	not applicable	Le Calvez, 1947
Elphidium crispum	obligatory	classically dimorphic	Lister, 1895; Schaudinn, 1895; Jepps, 1942

Ammonia tepida	facultative	Variable	Bradshaw, 1957; Schnitker, 1974; Goldstein & Moodley, 1993
'Tretomphalus' bulloides (=Rosalina globularis)	obligatory	classically dimorphic, but a terminal float chamber occurs in mature gamonts	LeCalvez, 1936b; Myers, 1943a
Discorbis patelliformis	obligatory	classically dimorphic	Myers, 1940
Discorbis mediterranensis	obligatory	classically dimorphic	LeCalvez, 1950
Discorbis vilardeboanus	?obligatory	classically dimorphic	Foyn, 1936; LeCalvez, 1950
Glabratella sulcata	obligatory	gamont larger than agamont	LeCalvez, 1950, Grell, 1958b, 1979
Rubratella intermedia	obligatory	not dimorphic	Grell, 1958a, 1979
Metarotaliella simplex	obligatory	not dimorphic	Grell, 1973, 1979
Metarotaliella parva	obligatory	not dimorphic	Grell, 1973, 1979
Rotaliella roscoffensis	obligatory	not dimorphic	Grell, 1957, 1979
Rotaliella heterocaryotica	obligatory	not dimorphic	Grell, 1954, 1979
Rotaliella elatiana	?facultative	gamont generally smaller	Pawlowski & Lee, 1992
Heterostegina depressa	biologically trimorphic	?trimorphic	Rottger *et al.*, 1990a
Amplistegina depressa	biologically trimorphic	Trimorphic	Harney *et al.*, 1998; Dettmering *et al.*, 1998
Planktonic taxa	?gamic	Unknown	See review in Hemleben *et al.*, 1989

Table 4.1: Table illustrating the record of reproductive cycles in benthic foraminifera
(After Goldstein, 1999)

It is clearly evident from the previous reports that the classic life cycle with alternating sexual and asexual generations is not always observed in many species of benthic foraminifera. Overall the lifecycle of foraminifera is more varied than in any other group of protists.

Every bit of information in this regard is important considering the fact that, studies on the reproductive traits of foraminifera are important in resolving many of the age old taxonomical problems. Almost all studies using foraminiferal proxies are directly or indirectly based very much on the taxonomy of this group, hence proper understanding of the reproductive cycles and the associated morphological variations in different species of foraminifera is very much a relevant topic of research. This is effectively possible in the laboratory cultures owing to the freedom of continuous and clear observation of the living specimens through various generations in response to the conditions provided.

During this research work, a number of different benthic foraminiferal species were cultured in the laboratory where they reproduced to give several generations (Plate 4.1) these include-

- *Rosalina leei*
- *Strebloides advena*
- *Pararotalia nipponica*
- *Discorbina concinna*
- *Cymbaloporetta plana*
- *Spiroloculina* sp.

There were some other species which could only be reared in the laboratory as reproduction was never observed under the given environment in the laboratory during the entire span of this study.

- *Trochammina* sp.
- *Ammonia tepida*
- *Ammonia beccari*
- *Massilina seccans*
- *Elphidium* sp.
- *Bolivina* sp.

Though the life span of only a few of these species were studied as a part of this work, the information on the viability of growing certain benthic foraminiferal species under laboratory cultures certainly helps to eliminate the misconception about handling the foraminiferal cultures and thus will be a stepping stone for the successors who wish to continue working on similar area of research.

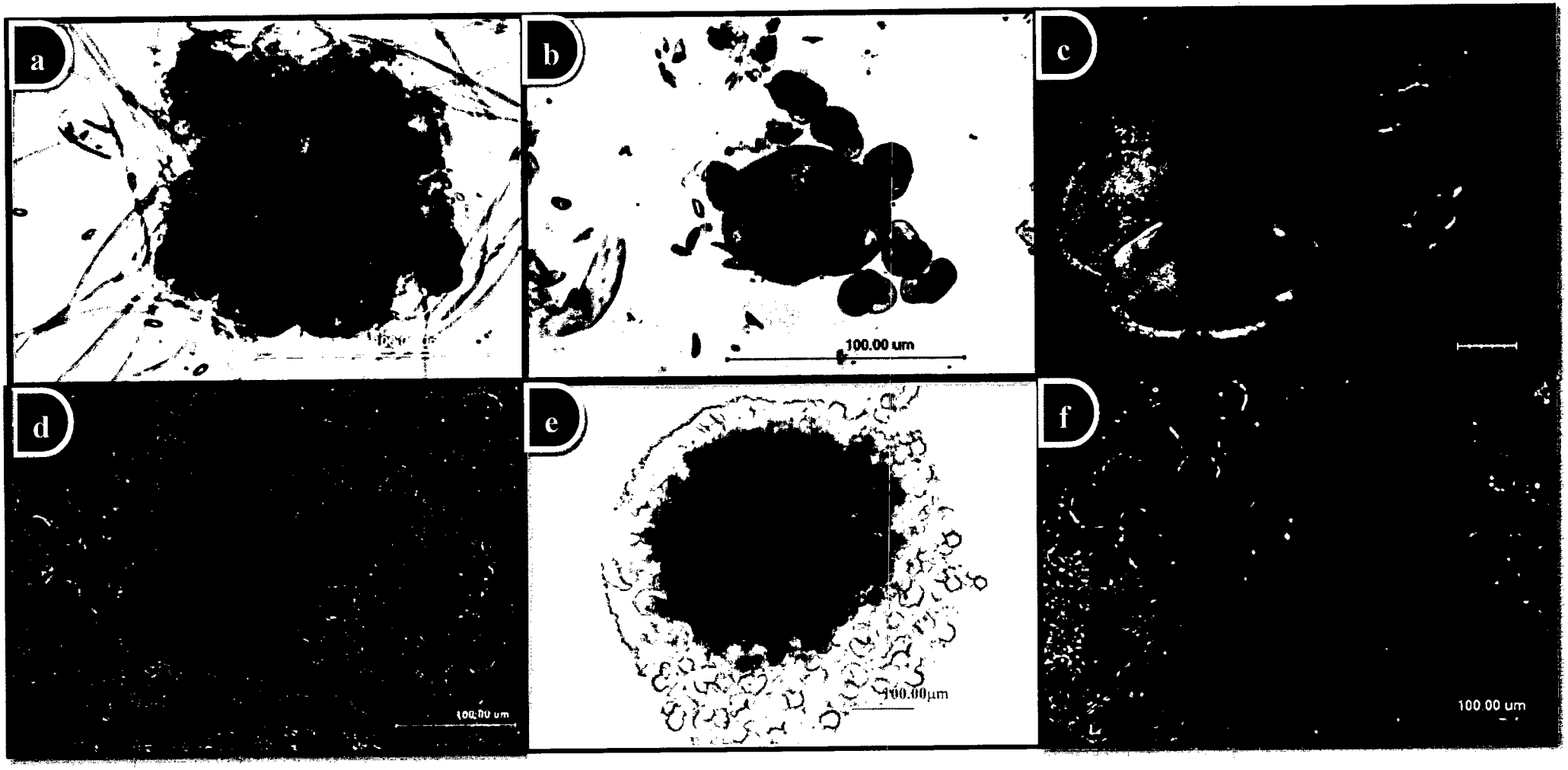


Plate 4.1: Reproduction in various benthic foraminiferal species under laboratory conditions
a) *Rosalina leei* **b)** *Strebloides advena* **c)** *Pararotalia nipponica* **d)** *Discorbina concinna* **e)** *Cymbaloporetta plana* **f)** *Spiroloculina* sp.

4.3.2. Life span studies on benthic foraminifera

Life span or duration of the reproductive cycle of foraminifera depends on the species and environmental conditions. Small species living in shallow mid-latitude waters may complete their reproductive cycle in several days, where as some larger species may live for two years. The studies of Myers (1938, 1941, 1943a) on *Elphidium crispum* are among the most extensive in this regard. The following table (Table 4.2) lists the lifespan/length of reproductive cycles of some of benthic foraminifera reported so far by previous workers (Boltovskoy & Wright, 1976). The list contains information of only 16 out of the thousands of extant benthic foraminiferal species and the list commences with 2 new additions from the present study which itself signifies the present study.

Species	**Author**	**Lifespan**
Spiroloculina hyaline	Arnold, 1964	2 weeks
Nubecularia lucifuga	Arnold, 1967b	few weeks
Rosalina sp.	Hedley & Wakefield, 1967	3-5 weeks
Streblus beccarii tepida	Bradshaw, 1957	4 weeks
Patellina corrugate	Myers, 1935a	41 days
Patellina corrugate	Berthold, 1971	40 days
Bolivina doniezi	Sliter, 1970	9-13 weeks
Heterostegina depressa	Rottger, 1972	8 months
Epistominella exigua	Boltovskoy & Lena, 1969	<1 year
Elphidium gunteri	Boltovskoy & Lena, 1969	<1 year
E. articulatum	Boltovskoy & Lena, 1969	<1 year
Quinqueloculina seminulum	Boltovskoy, 1969	1 year
E. macullum	Boltovskoy, 1969	1 year
Buccella frigida	Boltovskoy & Lena, 1969	1 year
Marginipora vertebralis	Ross, 1972	>1 year
Strebloides advena	**Present Study**	**17-19 days**
Rosalina leei	**Present Study**	**14-15 weeks**

Table 4.2: Table illustrating the record of length of reproductive cycle/ life span in benthic foraminifera
(After Boltovskoy & Wright, 1976)

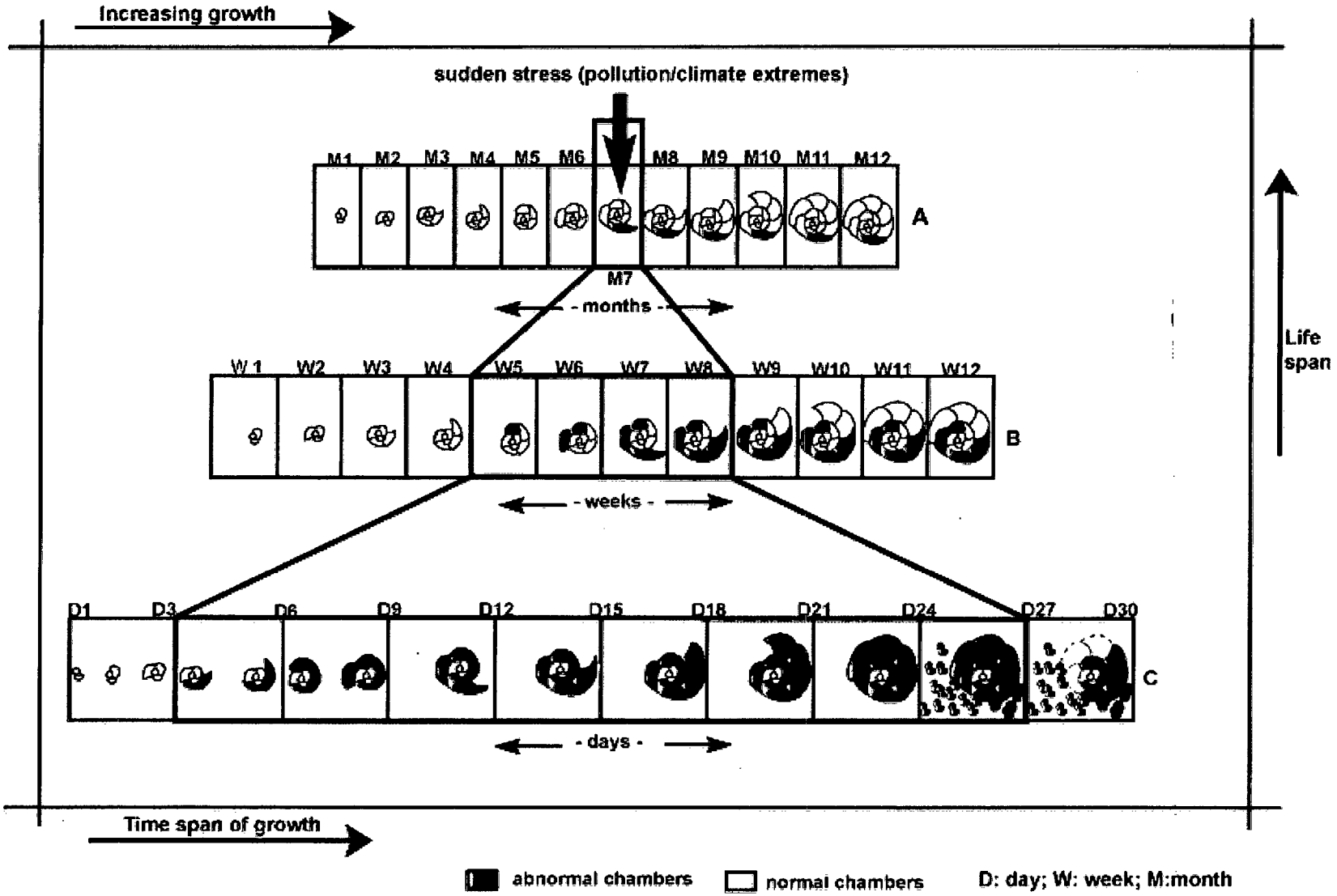


Fig. 4.6: Schematic diagram showing implication of life span of foraminiferal species in recording sudden/short term changes in environment.

A: species with life span ~ years, **B:** species with life span ~ months, **C:** species with life span ~ weeks.

4.3.3. Significance of shorter life span of certain species of benthic foraminifera

The species with shorter life span are better suited to investigate the differences between the long-term environmental changes and episodic stress events (Fig. 4.6). In case of species with life spans of the order of years, signatures of short term events will be recorded only in one or two chambers. Therefore abundance may not be affected by such short term changes. Similarly, in case of species with life spans of the order of months, only a few chambers or few specimens may be able to record such events. However the species with life spans of the order of a few weeks and with hard tests are the best suited to register such events. Many specimens will go through the complete life cycle during the span of even short term changes (natural/ anthropogenic). Their test will get preserved in sediments and exhibit the existence of short events in either composition (elemental or isotopic) or morphology of the test and even modification in the abundance may also change. The large number of tests in the sediments deposited in continuous layers therefore holds the key to understand such events in the past.

4.4 Experimental set-up

In order to study the growth and reproduction in the laboratory, the healthy living specimens of *Strebloides advena* and *Rosalina leei* (Fig. 4.7) were separated (as per the methods discussed in chapter 3) and kept in incubators with 12 hour light 12 hour dark illumination cycle, at several combinations of temperature and salinities. Additional feed was given in the form of living *Navicula* (Diatom) cultures at regular intervals (every third day). The growth pattern was monitored constantly and documented time to time under inverted microscope with the help of real time monitoring system and image analysis software. When the specimens reproduced (*Strebloides advena* as well as *Rosalina leei*), the juveniles along with the mother cell were transferred separately in to new culture dishes after one day of reproduction. The juveniles were maintained in the same conditions under which they were born. Similarly, many generations were monitored continuously to understand their lifecycle as well as life span in the laboratory conditions. The growth of the foraminiferal specimens was recorded in the form of chamber addition.

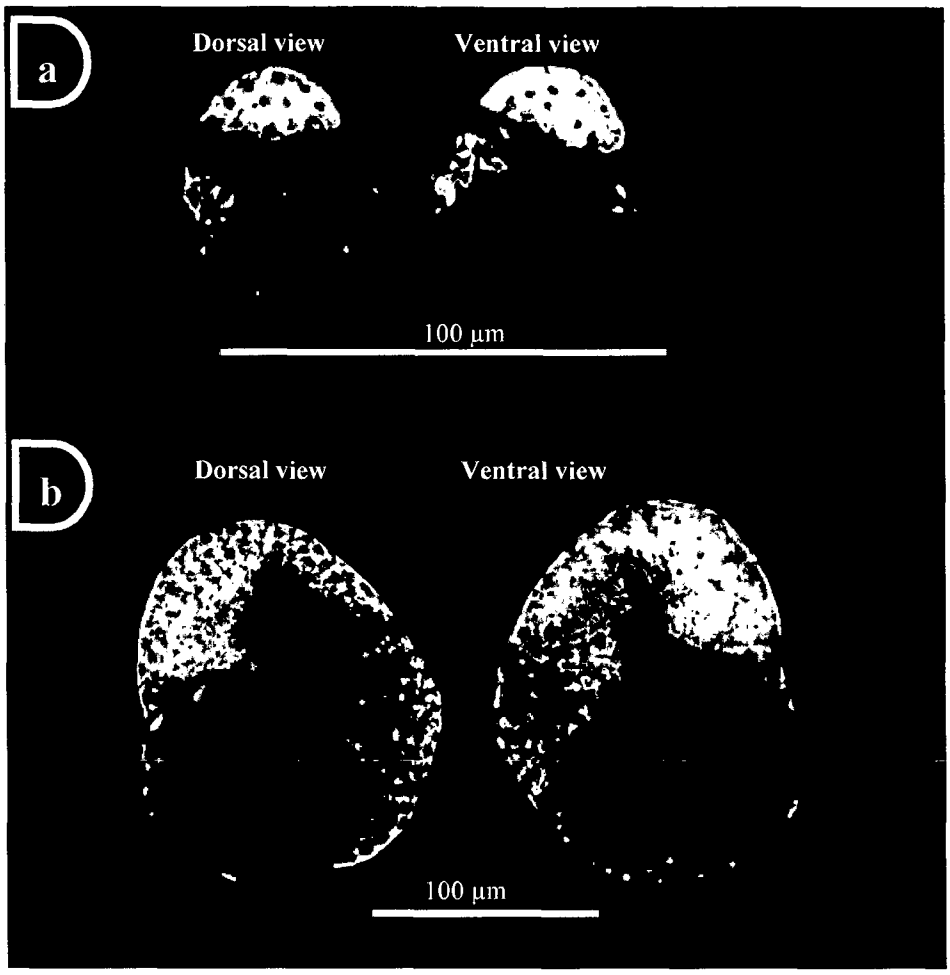


Fig. 4.7: Healthy living specimens of (a) *Strebloides advena* and (b) *Rosalina leei* from laboratory cultures

4.5 Results and discussion

During the study, the dimorphic cycle in both *Rosalina leei* as well as *Strebloides advena* was not observed; only asexual reproduction occurred in our laboratory cultures (Fig. 4.8). Parallel observations on different specimens on *Rosalina leei* and continuous observations through so many generations of *Strebloides advena* revealed the same in case of these species. A diagrammatic representation of the lifecycle of *Rosalina leei* and *Strebloides advena* as observed in our laboratory culture conditions is illustrated in figure 4.8. In this figure, two generations are included so as to exemplify that the asexual reproduction continued with each generation and in between no sexual reproduction was observed in these two species.

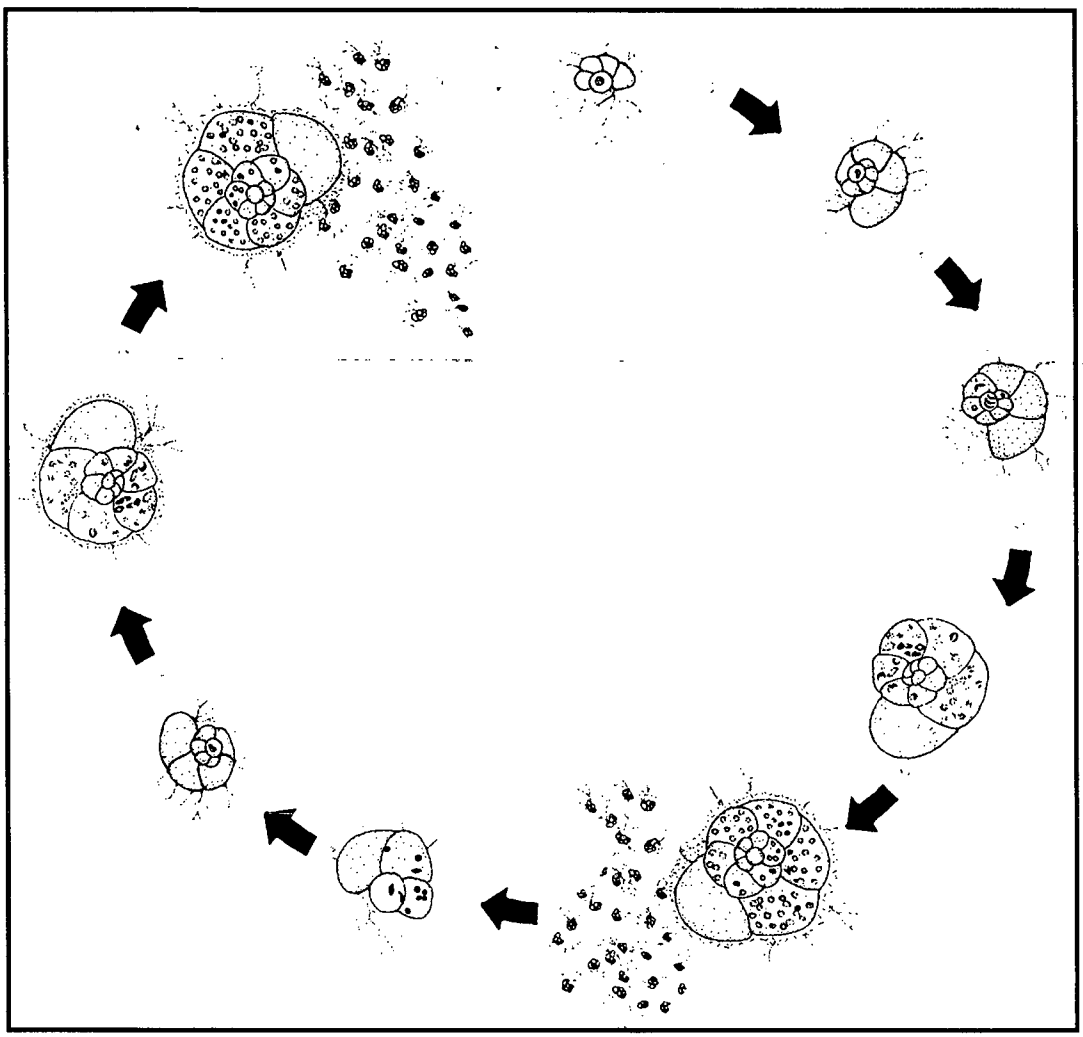


Fig. 4.8: The diagrammatic illustration of the life cycle of benthic foraminiferal species *Strebloides advena* & *Rosalina leei* as observed in the present study

4.5.1 Life cycle & Life span of *Rosalina leei*

Taxonomic status:

Order: Foraminiferida (Eichwald, 1830)

Suborder: Rotaliina (Delage & herouard 1896)

Family: Discorbidae (Ehrenberg, 1838)

Genus: *Rosalina* d'orbigny, 1826

Species: *Rosalina leei* (Hedley& Wakefield 1967)

4.5.1.1 Life cycle

Asexual reproduction was the only mode of reproduction observed in *Rosalina leei*. During the reproduction, juveniles come out of the mother cells with 2-3 (mostly 3 chambers) chambers partially filled with protoplasm. Pseudopodial activity was observed in the juveniles under high magnification and they were able to move away from the mother cell with the help of pseudopodia. The number of juveniles reproduced from different mother cells varies, but in general it was observed that 40-60 juveniles were formed from a single mother cell (Fig. 4.9). The number of the juveniles depends on the availability of living material i.e. the protoplasm; in that case it is to be assumed that bigger the mother, more number of juveniles produced under favorable conditions.

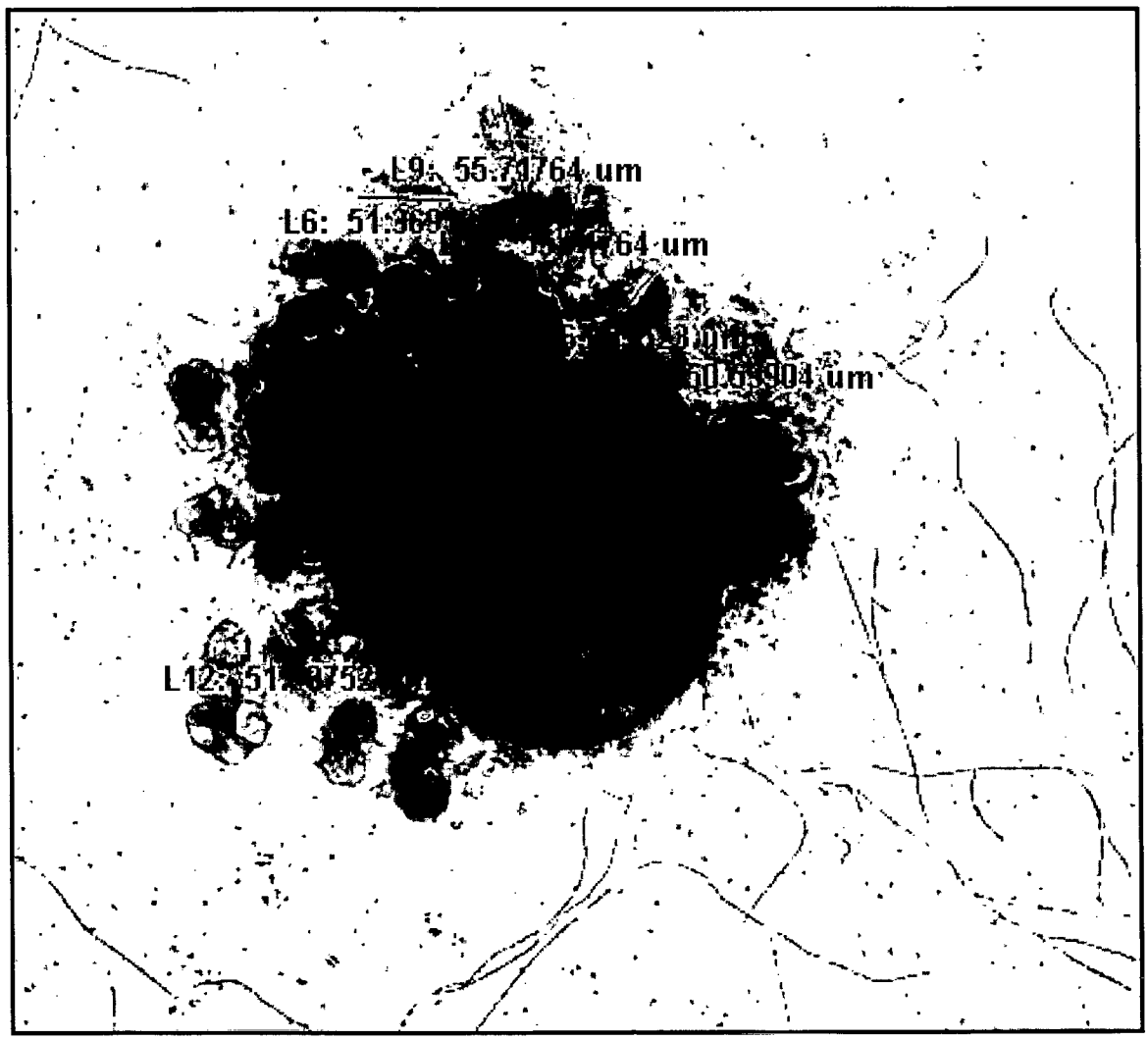


Fig. 4.9: ***Rosalina leei*: Mother specimen along with the juveniles during reproduction in the laboratory culture**

The size of the juveniles normally varies from 50-60 μm (Fig. 4.9) and a few of the juveniles at times attain bigger sizes. The juveniles receive protoplasm during their formation with in mother cell and protoplasmic coloration is clearly visible in initial chambers especially the proloculus. The juveniles show extensive pseudopodial activity and the length of the pseudopodia at times may reach several times the size of the individuals (Fig.4.10).

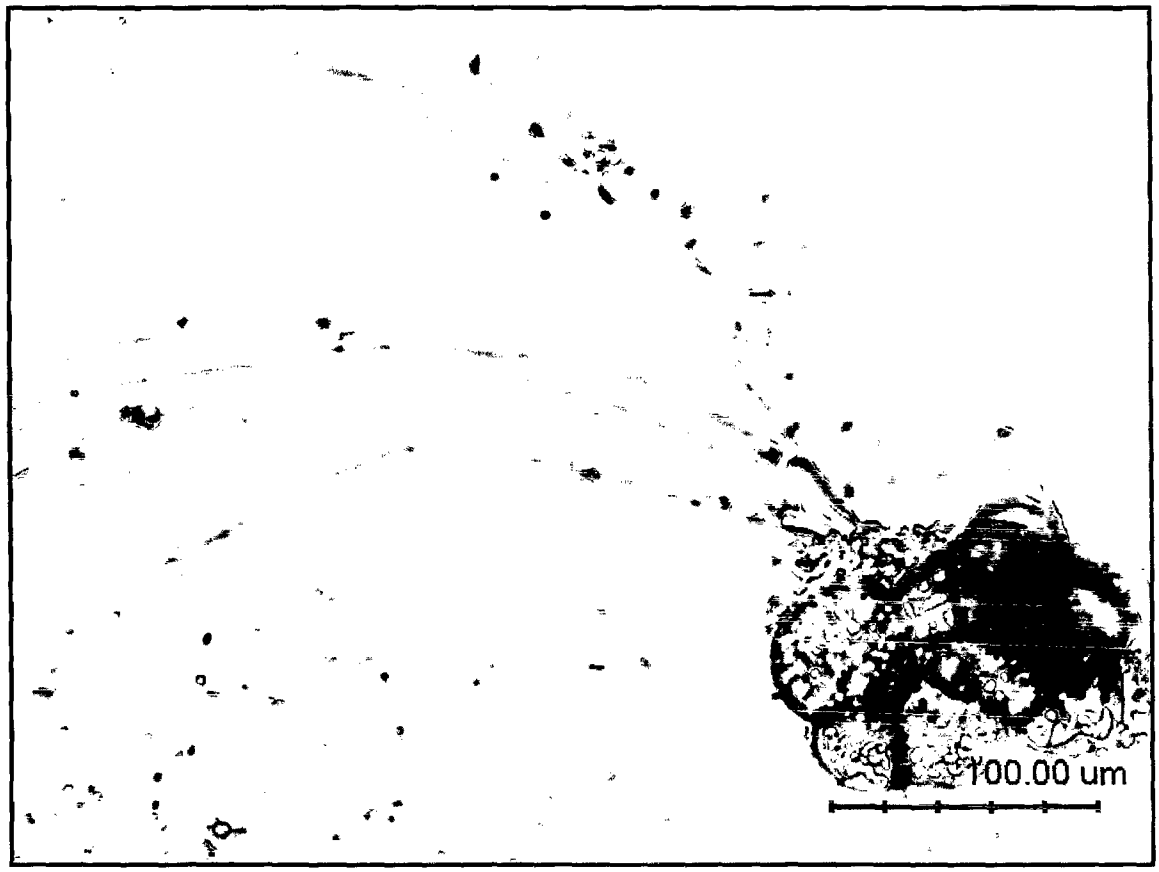


Fig. 4.10: Juveniles of *Rosalina leei* showing extensive pseudopodial activity; length of the pseudopodia reaches to 3-5 times the size of the juvenile test

Prior to reproduction, the specimen accumulates food particles around their test to form reproductive cysts (Fig. 4.11a). The juveniles come out of the mother cell, breaking a part of the test and at times the fragments of the mother test were seen in the culture dish. Since the entire protoplasm and at times part of the test material also is used up/utilized by the juveniles, reproduction typically terminates the life of the parent/mother specimen and an empty test remains in place of the mother specimen. Occasionally some protoplasm remains behind in the parent test for several days before decomposing.

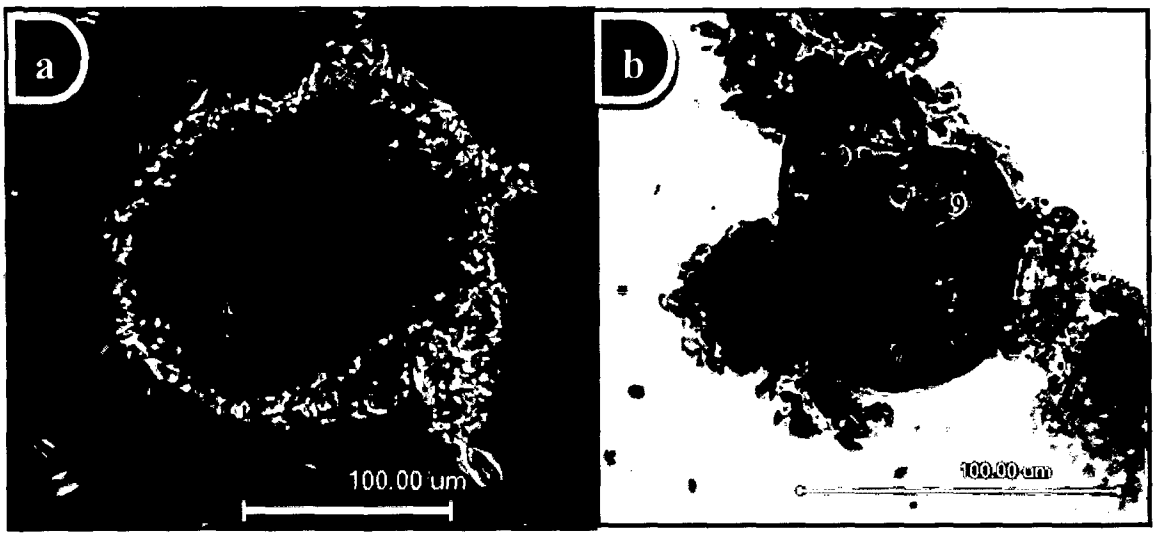


Fig. 4.11: *Rosalina leei*: a) Reproductive cyst formed by the specimen prior to reproduction. b) Mother specimen after reproduction, as an empty test devoid of protoplasm

4.5.1.2. Developmental stages of *Rosalina leei* in a complete life cycle as observed in laboratory cultures

In a detailed laboratory culture experiment conducted in our lab (Nigam *et al.*, 2008) wherein live specimens of *Rosalina leei* were subjected to different combinations of temperature (25°C, 30°C and 35°C) and salinity (25‰, 30‰ and 35‰), it was noticed that 25°C temperature and 35psu salinity is best suited for the growth of *Rosalina leei*. The study revealed that comparatively lower temperature and higher salinity increases the growth rate of *Rosalina leei* whereas the higher temperatures and lower salinity decreases the growth rate in this coastal benthic foraminiferal species. Though the optimum temperature-salinity conditions for the conducive growth was identified as 25°C temperature and 35‰, reproduction was not reported in any of the above mentioned combinations of temperature and salinity.

It was assumed that this species might be reproducing at narrow range which might be different from the combinations used for the study. In a previous report on *R. leei* by Hedley and Wakefield (1967) it is mentioned that this species reproduced at 17-20°C seawater temperatures. But the present study reports successful reproduction in *Rosalina leei* at 27°C temperature and 35 ‰ salinity.

The growth pattern of *Rosalina leei* was documented with the help of microphotographs taken with the help of a sophisticated inverted microscope which has live imaging facility and image analysis software. The growth rate can be determined either by the number of chambers added or from the linear addition to the maximum test diameter.

The microphotographs are arranged sequentially in order to get a clear understanding of the growth in *R. leei* in terms of chamber addition (Plate 4.2). It is evident from the observations that the growth rate is quite fast in the initial stages of growth. Rate of chamber addition becomes slower as the organism grows larger. Once the specimens attain the maximum size, it remains idle/dormant for some time preparing itself for the reproduction. The total number of chambers formed by *Rosalina leei* during its life varied in different specimens and normally ranged between 10- 16, though exceptions were seen (Fig. 4.11b). Similarly the maximum size attained by the specimens varied from 270-340 μm at maturity other than the exceptional cases where specimens either attained much bigger/smaller sizes in the cultures. For this reason, the maximum size attained or the maximum number of chambers formed by this species is expressed as a range rather than a particular value.

The chamber formation in *Rosalina leei* revealed that prior to the addition of new chamber; the juvenile specimens use their pseudopodial network to accumulate foreign particles, mainly the food particles to form an outline in the form of the new chamber within which the new chamber is formed. In Fig 4.12 it is clear that this mass of foreign particles does not cover the entire test as it happens during the reproduction, but only forms over the region where the new chamber has to form in this case the 4th chamber. The newly formed chamber is distinct with its transparent colour compared to the previous chambers. Later on the protoplasm from the previous chambers flows in to fill the new chamber.

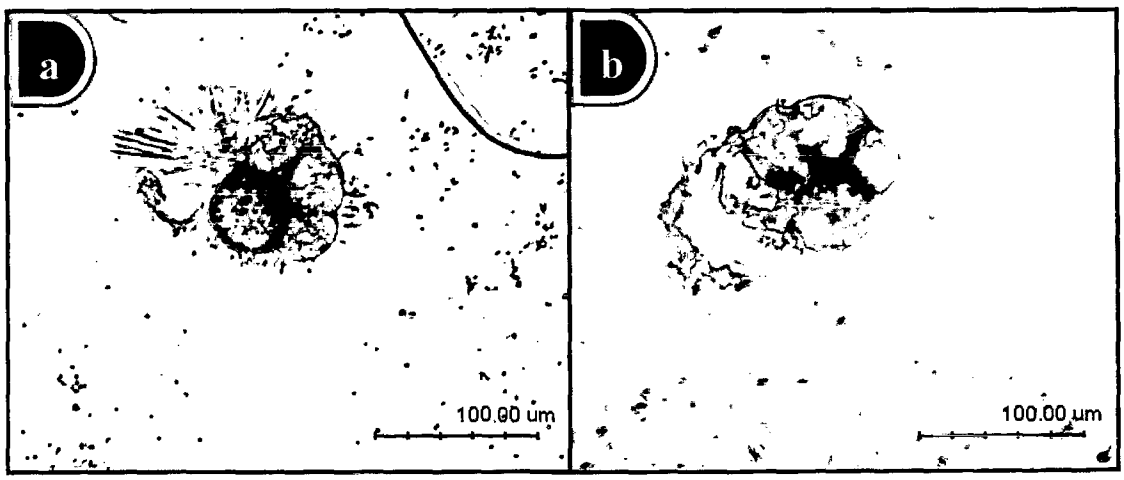


Fig. 4.12: (a) Extended pseudopodial network to accumulate the foreign particles (b) New chamber formed within the outline formed of foreign particles

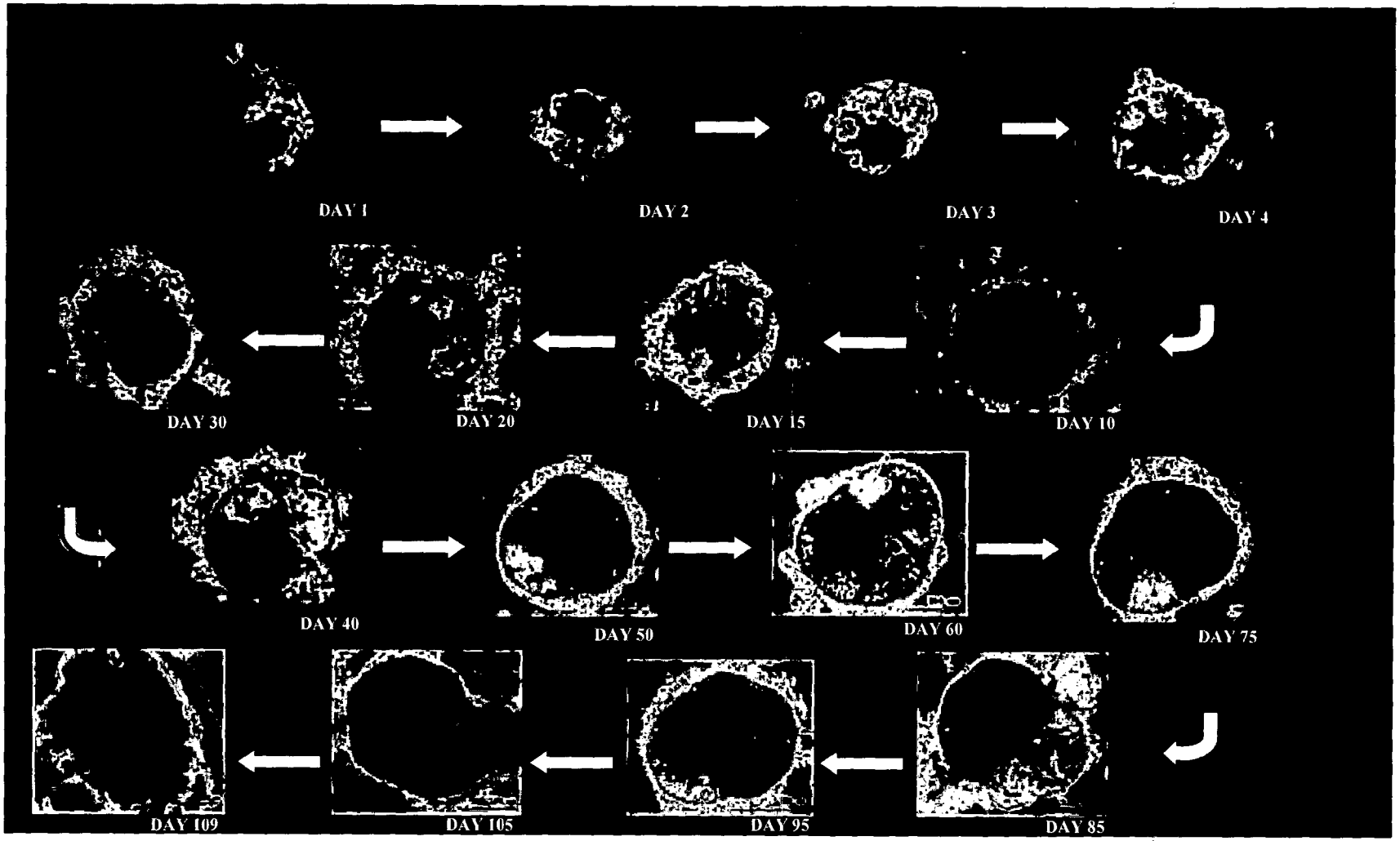


Plate 4.2: Growth stages/ chamber formation in *Rosalina leei* from laboratory cultures

4.5.1.3. Life span

From the parallel observations made on several specimens in the laboratory, only asexual mode of reproduction was observed (as described under life cycle of *Rosalina leei*). In all the cases, *Rosalina leei* completed its life cycle within 105-109 days interval ((Fig. 4.13).). Since reproduction terminates the life of the mother specimen, lifespan coincides with the reproductive span of this species. Hence life span can also be described as the length of the reproductive cycle of the benthic foraminiferal species *Rosalina leei* and it is noticed to be 105-109 days under the given laboratory conditions.

Previously a species namely *Rosalina floridana* was studied by Lee *et al.* (1963) who reported that this species has classical dimorphism with alternating sexual and asexual phases during its lifespan. Later on Hedley & Wakefield (1967) modified the taxonomic status of *Rosalina floridana* and proposed it to be *Rosalina leei* and reported the life span of *Rosalina leei* as 3-5 weeks through a series of apogamic reproduction devoid of any dimorphism from the laboratory observations on specimens collected from Plymouth, England and Wellington, New Zealand. However Boltovskoy & Wright (1976) have not agreed with this taxonomic modification and kept it open as *Rosalina* sp. while listing the previous record on the length of reproductive cycles in benthic foraminifera. The specimen studied as a part of this work has most of the characteristics of *Rosalina leei* and from the present study a life span of 14-15 weeks was noted. It appears that this species is cosmopolitan as it is reported from both temperate (by Hedley & Wakefield, 1967) to tropical zones (by Saraswat *et al.*, 2004; Nigam *et al.*, 2008 and also the present study).

The difference in the life span may possibly be attributed to the difference in geographic zones of the samples used for the studies; England as well as New Zealand falls in temperate zones where as the sample for the present study is from a tropical zone. It should also be noted that since the present report of the life span of *Rosalina leei* is based only on two consecutive laboratory generations, more careful observations are required to confirm the life span of the species.

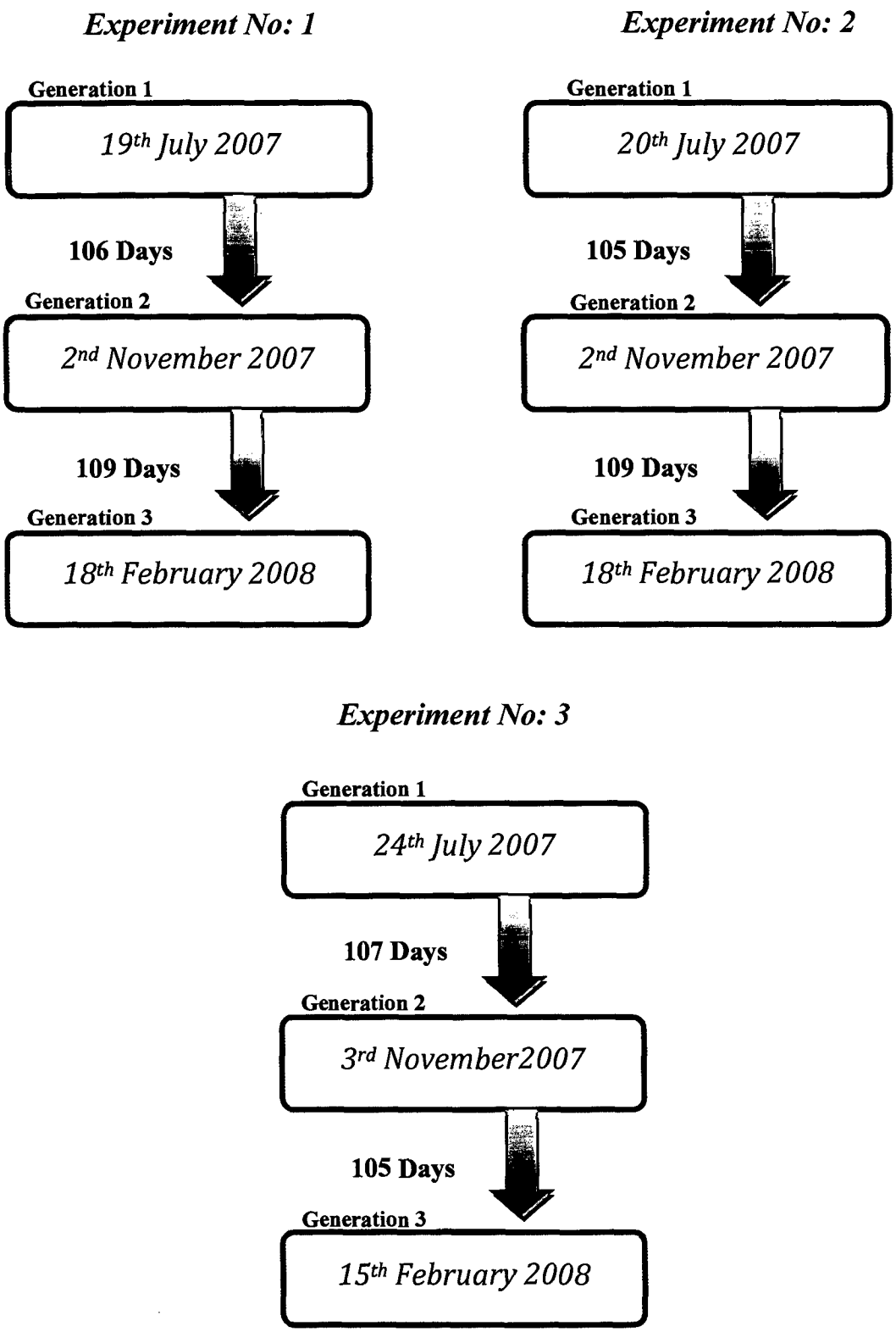


Fig. 4.13: Diagram illustrating the dates of reproduction of *Rosalina leei* in the laboratory cultures. The arrow depicts the successive generations

4.5.2 Life cycle & life span of *Strebloides advena*

Taxonomic status:

Order: Foraminiferida (Eichwald, 1830)

Suborder: Rotaliina (Delage & herouard 1896)

Family: Discorbidae (Ehrenberg, 1838)

Genus: *Strebloids* (Bermudez & Seiglie 1963)

Species: *Strebloids advena (Cushman 1922)*

4.5.2.1 Life cycle

Asexual reproduction was the only mode of reproduction observed in the laboratory throughout the number of generations studied. The first signs of reproduction were visible several days earlier than the release of the offsprings. The offsprings per reproducing adult specimens varied from 15-20 in numbers. The pseudopodial activity was observed in many of the offsprings at high magnification, and they were very actively moving around in the media and over the algal matter available in the culture dish. The protoplasm was reddish brown in colour and apart from that fluorescent orange colored vacuoles were seen in all the juveniles and also scattered in the periphery of the mother test. On careful observation it was observed that the juveniles acquired these masses along with the protoplasm from the mother cell as it is visible from the photograph. It is possible that these are the algal symbionts they acquired from the mother cell. Reproductive cyst formed from the food particles accumulated around the test prior to reproduction was not commonly seen for this species though it was noticed for many of the other benthic foraminiferal species in our laboratory.

During reproduction, the juveniles consumed the entire protoplasm of the mother cell the mother cell became totally empty and died out with the reproduction. The number of juveniles produced from a mother cell thus depends on the availability of the amount of protoplasm.

It was observed that smaller specimens produced less number of juveniles and more number of juveniles was produced from bigger specimens. For e.g. specimens of the order of 65.58 μm size produced less than 10 juveniles whereas mother specimens of the order of 80-81 μm size produced 15-20 juveniles, each of which was 25 μm to 32 μm in size at the time of its birth (Fig. 4.14).

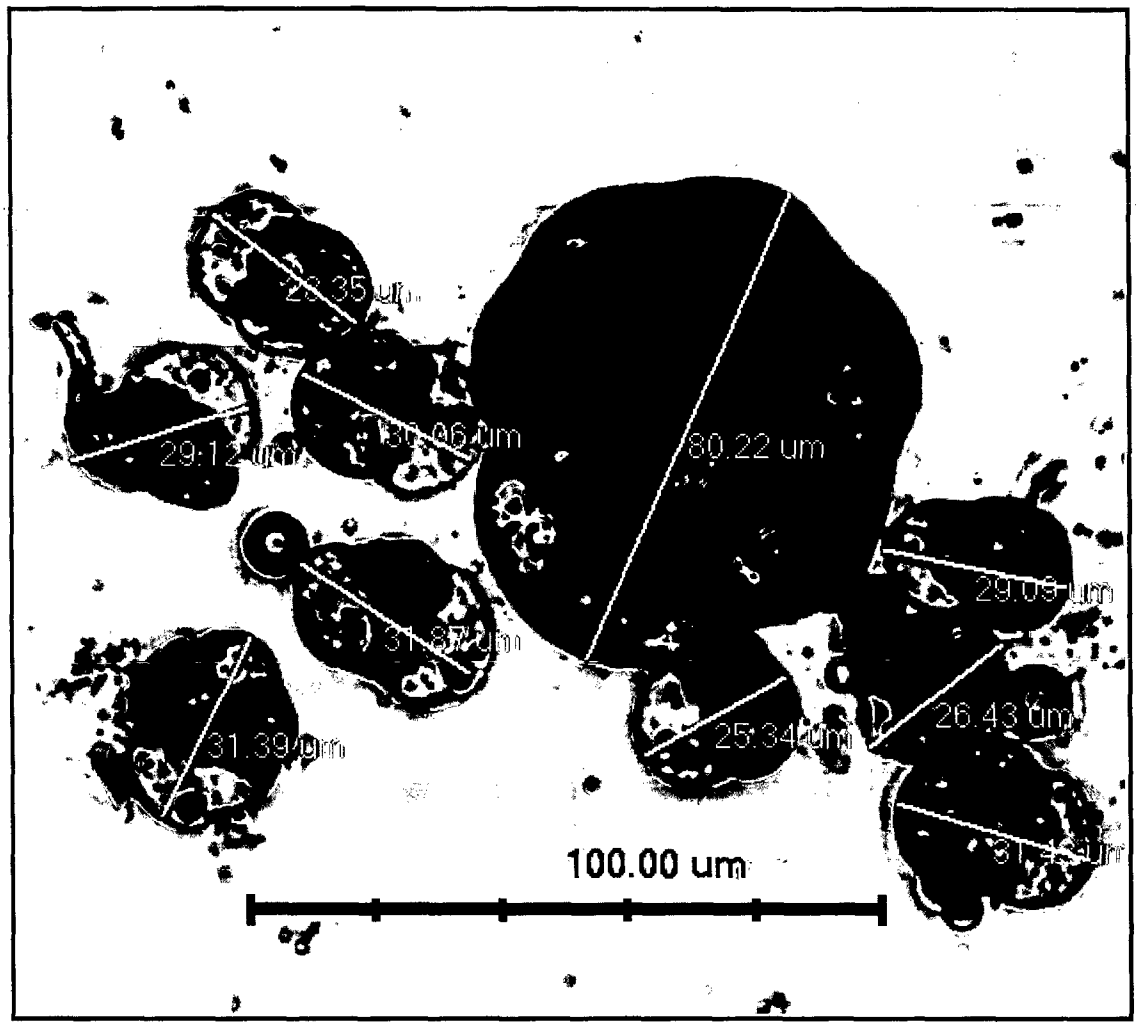


Fig. 4.14: *Strebloides advena*: Mother specimen along with the juveniles during reproduction in the laboratory culture

4.5.2.2. Developmental stages of *Strebloides advena* in a complete life cycle as observed in laboratory culture conditions

Out of several combinations of salinity and temperature conditions given to *Strebloides advena* in the laboratory with 12 hour light 12 hour dark illumination cycle and additional feed of mixed diatom culture, best response was observed for the ones maintained at 36‰ salinity and 30°C temperature.

Strebloides advena formed 11-14 chambers at maturity and grew to the maximum size of 90 μm in culture; the lower limit of this range varied between 65 μm-70 μm. The size of the mature specimens remained more or less the same through all the generations. The growth of the *Strebloides advena* during a complete life cycle is

presented with chronologically arranged microphotographs of the specimens taken at the progressing stages of growth in the laboratory (Plate 4.3).

The juveniles were born with 3-4 chambers, further chambers were added after the birth. Chamber addition was faster during the initial 5-6 days when they add 5-6 chambers out of the total 11-13 chambers they have at maturity. Thereafter the chambers were added at a relatively slower rate and by day 12 they have already completed the chamber addition (11-13; vary in individuals). The signs of reproduction were clearly visible through the test; the test was entirely filled with protoplasm and quite a few granular masses were visible in the protoplasm. By day 15, the juveniles were clearly visible within the mother cell. On day 19 (in this particular specimen) the reproduction took place, and it took almost a full day to complete the whole reproduction (till the last baby is out of the mother cell).

Reproduction usually terminated the life of the mother specimens in all the cases. The entire protoplasm of the mother cell was consumed by the juveniles leaving an empty test, totally devoid of protoplasm, either completely broken in to fragments or at times broken only on the ventral side of the last chamber depending how the juveniles came out (Fig. 4.15a,b).

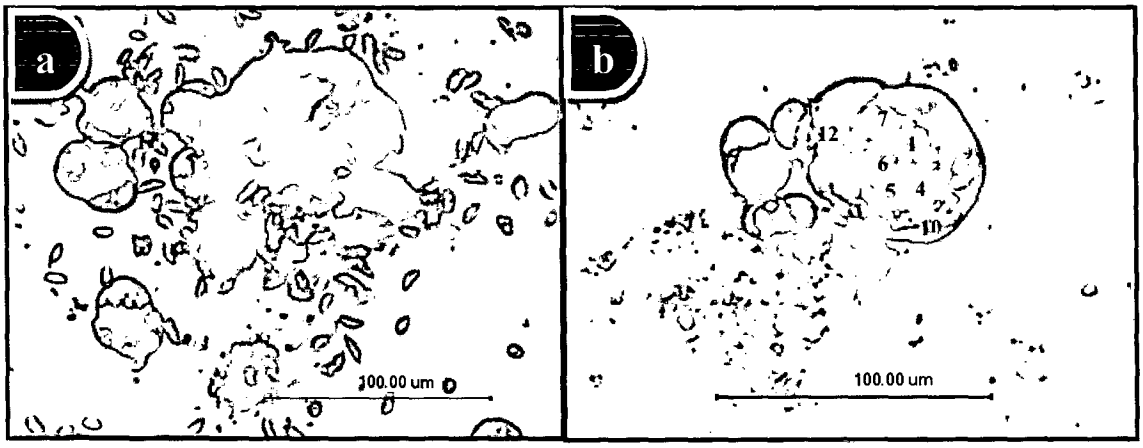


Fig. 4.15: ***Strebloides advena*****: Fate of the mother specimen after reproduction**
a: completely broken **b:** partially broken

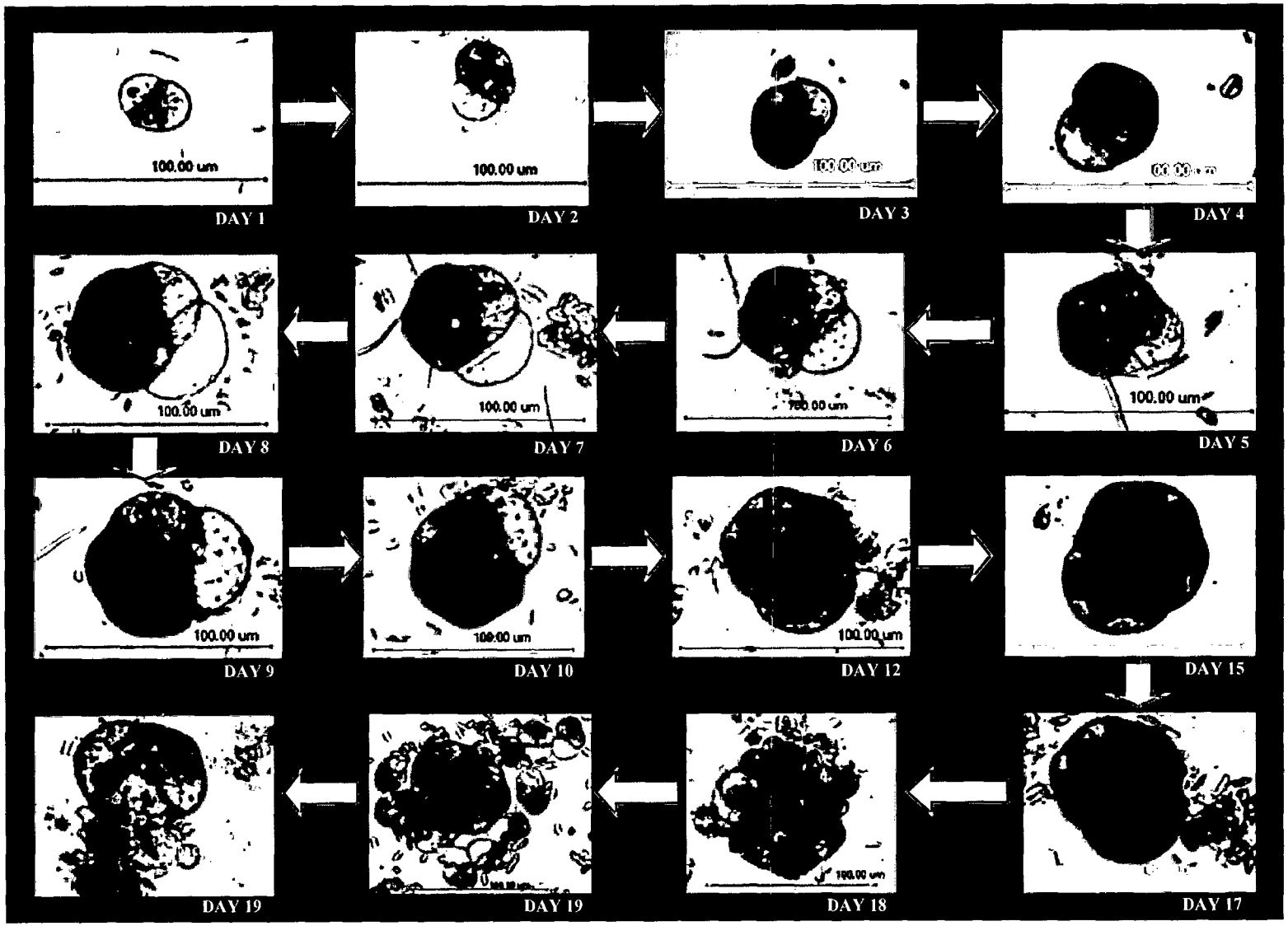


Plate 4.3: Growth stages/ chamber formation in *Strebloides advena* from laboratory cultures

4.5.2.3. Life span

Several consecutive generations were observed in the laboratory. In all the generations *Strebloides advena* completed its life cycle within 17-19 days (Fig. 4.16). Since reproduction terminates the life of the mother specimen, life span coincides with the reproductive span of this species. Hence life span can also be described as the length of the reproductive cycle of the benthic foraminiferal species *Strebloides advena* and it is noticed to be 17-19 days under the given laboratory conditions. This is the first report on the life span of this particular species and the very short life span of the order of 17-19 days is confirming the assumptions made by previous workers like Myers (1935b, 1937) and Arnold (1948, 1954a) that a smaller species will have shorter life span.

4.6. Conclusions

4.6.1. *Rosalina leei*

- Under the given laboratory conditions, *Rosalina leei* underwent only asexual reproduction in all the three consecutive generations.
- The number of juveniles formed from a single mother cell varies from 40-60 in number and the juveniles were born with 3-4 chambers.
- Prior to reproduction *Rosalina leei* made a reproductive cyst surrounding the test with the pseudopodial network.
- Life span of *Rosalina leei* was observed to be 105-109 days.

4.6.2. *Strebloides advena*

- Under the given laboratory conditions, *Strebloides advena* underwent only asexual reproduction in all the 7 consecutive generations.
- The number of juveniles formed from a single mother cell varied from 15-20 in number and the juveniles are born with 3-4 chambers
- In *Strebloides advena* the process of making reproductive cyst was not noticed prior to reproduction
- Life span of *Stebloides advena* was observed to be 17-19 days.

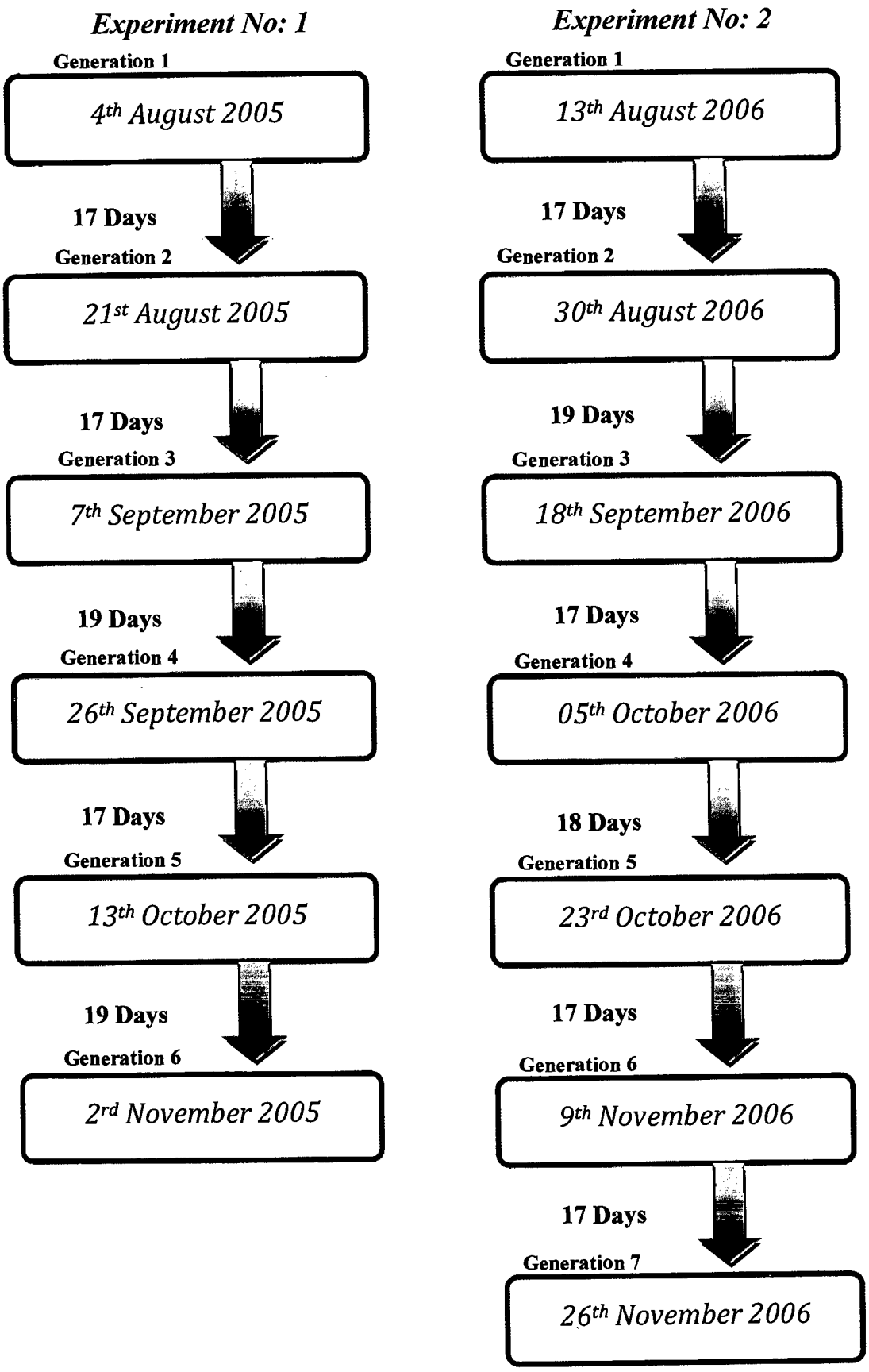


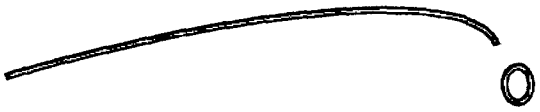
Fig. 4.16: Diagram illustrating the dates of reproduction of *Strebloides advena* in laboratory cultures. The arrows depict the successive generations

4.7. Significance of the study

Laboratory studies on various protozoa other than foraminifera have shown that if hundreds of asexually reproduced generations are created uninterrupted by a sexual generation, the broods begin to show signs of decay, the periods between reproductions (length of reproductive cycle) increases, the resistance of the offspring diminishes and the organisms perish. These observations have not held true for those foraminifera which repeatedly reproduce without a sexual generation. Some of the studies have reported a reduction in the size through generations, but the same was attributed to the artificiality of the conditions and the possible lack of sufficient food for the growth in the overcrowded culture (Boltovskoy & Wright, 1976). In the present study, any notable variation in the length of the reproductive cycle or any reduction in the size of the specimens with generations was not observed. It may be possible that the foraminifera possess some mechanism as yet unknown, which maintains their vital processes in equilibrium despite recycling the same genetic material. More studies in this direction may help resolve the existing problems related to the ambiguity on the life processes of the foraminifera in response to various environmental conditions and the related puzzles on the taxonomy based on the test morphology. This in turn will refine the use of both fossil as well as living foraminifera as a proxy for various environmental and paleoenvironmental studies.

CHAPTER 5

FORAMINIFERAL RESPONSE TO DIFFERENT OXYGEN LEVELS



Chapter 5

Foraminiferal response to different oxygen levels

"I'm a great believer in luck, and I find the harder I work, the more I have of it."

- Thomas Jefferson

5.1. Introduction

Oxygen content of bottom waters is one of the main environmental factor controlling foraminiferal distribution pattern in the sediment (Jorissen *et al.*, 1995). The amount of dissolved oxygen in seawater (including the values of super saturation) varies between 0-8.5 ml/l, the usual range being 1-6 ml/l (Tait, 1981). As per the classification of aquatic environments and the corresponding biofacies by Tyson and Pearson 1991, there are four zones in the oceans based on the dissolved oxygen concentrations (a) oxic (aerobic biofacies, with 8.0-2.0 ml/l O_2), (b) dysoxic (dysaerobic, with 2.0-0.2 ml/l O_2), (c) suboxic (quasi anaerobic, with 0.2-0.0ml/l O_2) and (d) anoxic (anaerobic, with 0.0 ml/l O_2). The more general physiological or ecological term 'hypoxia' indicates a degree of oxygen depletion that would induce a severe stress in marine organisms without necessarily implying a specific threshold value (Tyson & Pearson, 1991). The zone in the oceans, where concentration of dissolved oxygen is least, due to excessive use of available oxygen by organisms for respiration and organic matter (to degrade itself) and lesser supply of O2 due to reduced circulation of water is referred to as Oxygen Minima Zone or OMZ (Sen Gupta & Machain- Castillo, 1993).

Earlier field investigations interpreted past oxygen-related environments based on subsurface foraminiferal data (Alve, 1991; Barmawidjaja, 1995; Rabalais, 1996; Sen Gupta *et al.*, 1996). Benthic foraminifera were reportedly affected by the oxygen minima conditions that lead to variation in abundance of foraminiferal assemblages (see Bernhard & Sen Gupta, 1999, for review). So far it has been reported that the foraminiferal abundance is negatively correlated with the bottom water oxygen conditions (Bernhard, 1992). But on the other hand it had been reported earlier by Phleger & Soutar (1973) that oxygen is not a limiting ecological factor for benthic foraminifera in the low oxygen environments of Baja, California. Douglas *et al.*, (1980) and Thompson (1982) noted high standing stocks of benthic foraminifera in the anoxic environment of Santa Barbara and

Santa Monica basins due to the lack of macrobenthic predators. The arguments and the counter arguments continue. However it is difficult to attribute typical responses of foraminifera directly to changes in dissolved oxygen, because in nature, different important environmental factors, like food content and oxygen concentration work together and the influence of each factor cannot be delineated. Additionally, various foraminiferal species respond differently to different conditions, because each has its' own physiological ability to either adapt or retract. Laboratory experiments provide a good opportunity to analyze the response of different foraminiferal species to experiment simulated variations on one single environmental factor. Quite a few attempts have been made in the past using varied experimental modes (Moodley *et al.,* 1998b; Alve. & Bernhard, 1995; Geslin *et al.,* 2004). The Oxygen Minima Zone (OMZ: ~125-1500 m) from west coast of India is well known. However it is coastal hypoxia (~50-60m) which is a recent finding (Naqvi *et al.,* 2000) and less understood along the west coast of India (Nigam *et al.,* 2009) with special reference to anthropogenic changes. Thus laboratory experiments could give significant clues towards the foraminiferal response to the oxygen minima conditions which can help to understand the processes leading to OMZ. But no such experiment has been carried out with foraminifera from the Indian waters. The main objective of this study was to observe the effect of changed oxygenation on the vertical distribution of foraminifers. Sediment samples for the present study were collected off Ratnagiri, west coast of India (details of which are explained in chapter 3). The oxygen concentrations was changed in an experimental set up, while other environmental parameters including temperature and salinity were kept stable; the complete details of the experiment is given below.

5.2. Experimental set-up

A plastic liner with 8 inch diameter was inserted into the box core sediments slowly to a depth of ~10 cm. Care was taken that the fluffy layer is not disturbed and the liner was immediately covered with acrylic lids. This served as an experimental core, containing a minimum of 10cm of bottom sediments and 20 cm of the overlying water column (Fig.5.1).

Of the three such cores collected for this experiment, two cores served as experimental cores and were placed in the incubator. The third core was considered as the control core and taken to the cold room, sub-sectioned immediately at intervals 0-1, 1-3, 3-5, 5-7 and 7-

10 cm. These subsamples were stained with Rose Bengal-formalin solution to distinguish live (stained) and dead foraminifera in order to generate background data to record the vertical distribution of live foraminifera in normal field conditions.

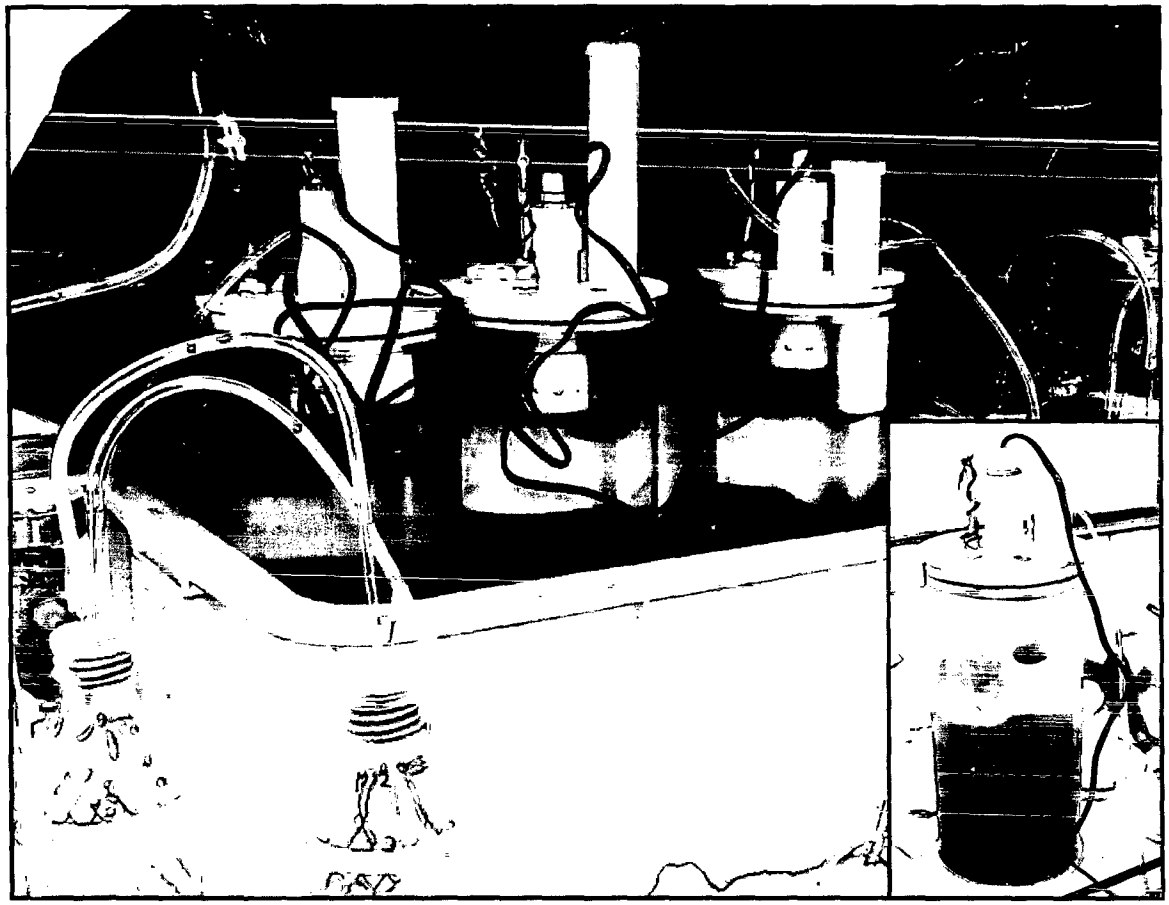


Fig. 5.1: Experimental Set up to observe the effects of different levels of oxygen

The oxygen concentration in the bottom waters was found to be 68 µmol/l and the temperature was recorded as 11°C. Hence the temperature of the incubator was set to 11°C and allowed to stabilize for over 6 hours. Insitu sediment oxygen profiling could not be done due to unavailability of the highly sophisticated oxygen sensitive electrodes.

Of the two experimental cores in the incubator, one of the cores was subjected to high oxygen concentration by aeration using an aquarium pumping system. In the other core, low oxygen conditions were attained by continuous and steady introduction of nitrogen (98%) into the overlying water column.

Electrically motored magnetic stirrers were inserted in both core liners to assure well circulation of the gases in the water column, taking care that the fluffy layer is not disturbed. This complete set up was transported to the National Institute of Oceanography,

the onshore laboratory at Goa. A temperature controlled container lab (Temporarily imported from NIOO, Netherlands) was then set up and used further. The experimental set up was then transferred from the incubator to the thermo lab, which offered more working space. The two experimental cores were covered with a black sheet to simulate ocean bottom illumination conditions of the sampling location. Salinity was measured regularly and also at the end of the experiment as 36.15 psu. To maintain continuous food supply, a pipette was used to uniformly spread bulk diatom culture over the sediment surface at regular intervals. After incubation for 15 days, the two experimental cores were also sub-sectioned at same intervals as the background core and each layer was stained with Rose Bengal/formalin. The stained specimens were counted in each subsection of all the three cores. Graphs were plotted to show the comparative data (Fig.5.2).

5.3. Results and Discussion:

The oxygen concentration in the water column in the experimental cores just before sub-sectioning was 3.6 μmol/l in the low oxygen core and 303.9 μmol/l in the high oxygen core. Majority of the total assemblage comprised *Fursenkoina, Bolivina, Nonion, Rotalia* and *Reophax*, listed according to degree of abundance. A small number of *Bulimina, Cancris, Ammotium, Globobulimina* were also present which have been clubbed as 'others'. Many soft-shelled forms also exist, but have not been considered in the present study as they have no fossilization potential and thereby of no geological significance. This experiment only presents the effect of oxygen manipulations on major foraminiferal genera. The similar horizons of the three differently treated cores have been compared to delineate effects of oxygen manipulations on foraminiferal distributions with sediment depth. No live foraminifers were found in the sub-sections beyond 5 cm. Identification of the specimens to the species level was not possible due to the following reasons (1) The specimens were extremely small, undoubtedly juveniles, (2) Picking for live required wet picking, (3) Prolonged exposures of samples to warmer temperatures caused bleaching of stain and also decay of specimens, disfiguring the identifying features.

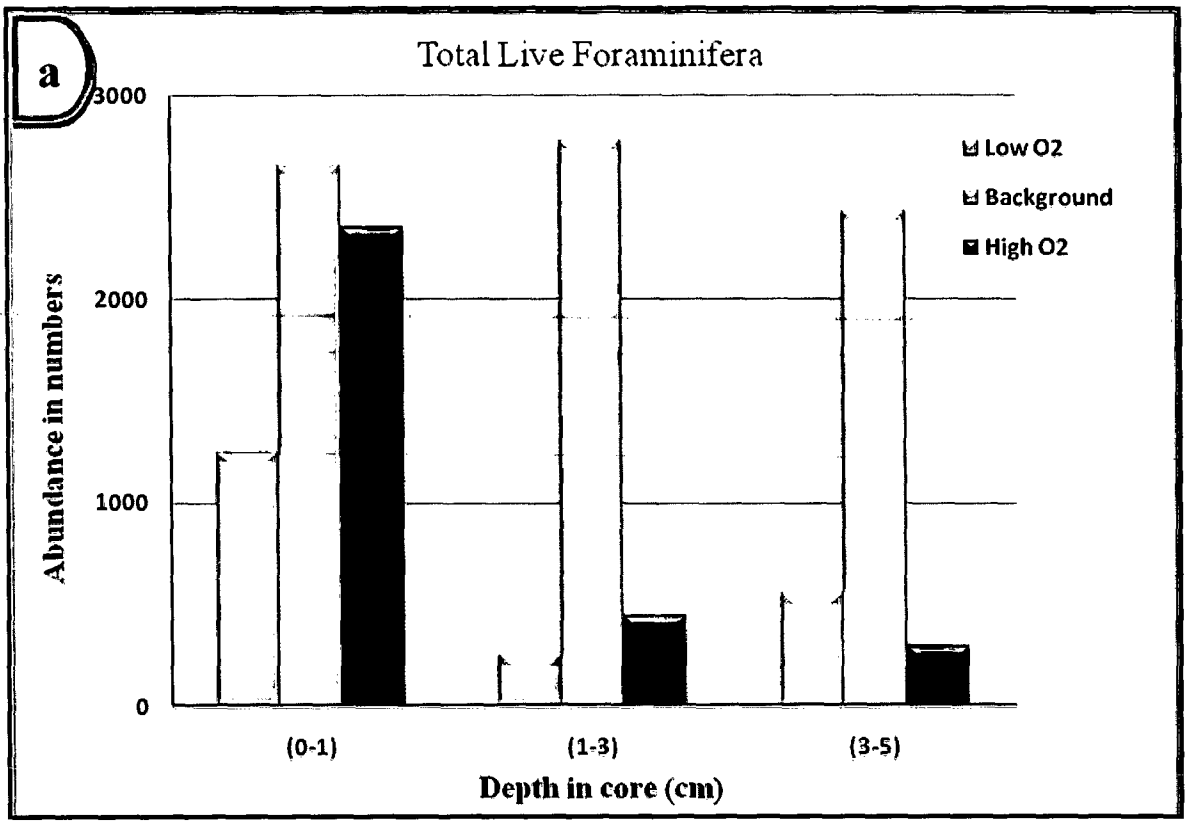
The response of the foraminifers to drastic alteration in oxygen conditions may be explained (Fig. 5.2) as follows: The Live Total Foraminiferal Number (Live TFN) is lower than normal in both cases of lowered and increased oxygen (Fig. 5.2a). This indicates that oxygen is a very critical factor for foraminifera. *Fursenkoina* (Fig. 5.2b) seem to be sensitive and die out with reduction in oxygen and congregate at the surface with increase

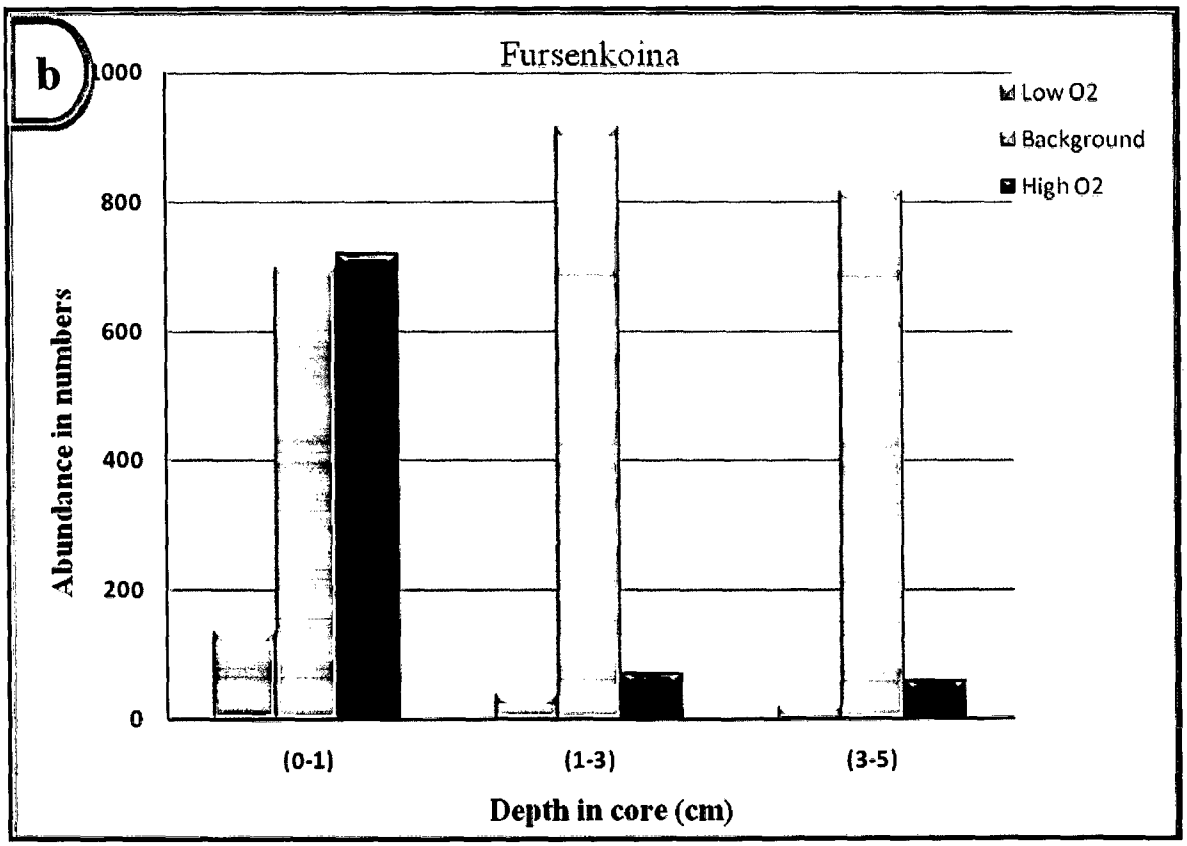
in oxygen. *Bolivina* (Fig. 5.2c) numbers fall drastically with any change in oxygenation. *Nonions* (Fig. 5.2d) also adapt along with changing oxygenation. They are seen to migrate to the surface (from oxygen depleted to more aerated zones) on lowering oxygen. This is in confirmation with Alve & Bernhard (1995). In contrast *Rotalids* (Fig. 5.2e) die out with; both increased or decreased oxygen concentration. *Reophax* (Fig. 5.2f) is agglutinated elongated form, which do not have surface pores. Thus, its resistance to low oxygen is evident by its tendency to move towards the surface on oxygenation. This explanation is supported by the widely accepted idea that foraminiferal pores are conduits for oxygen supply to the intracellular milieu (Berthold, 1976; Naqvi *et al.,* 2000) and that larger, more numerous pores could presumably provide more oxygen.

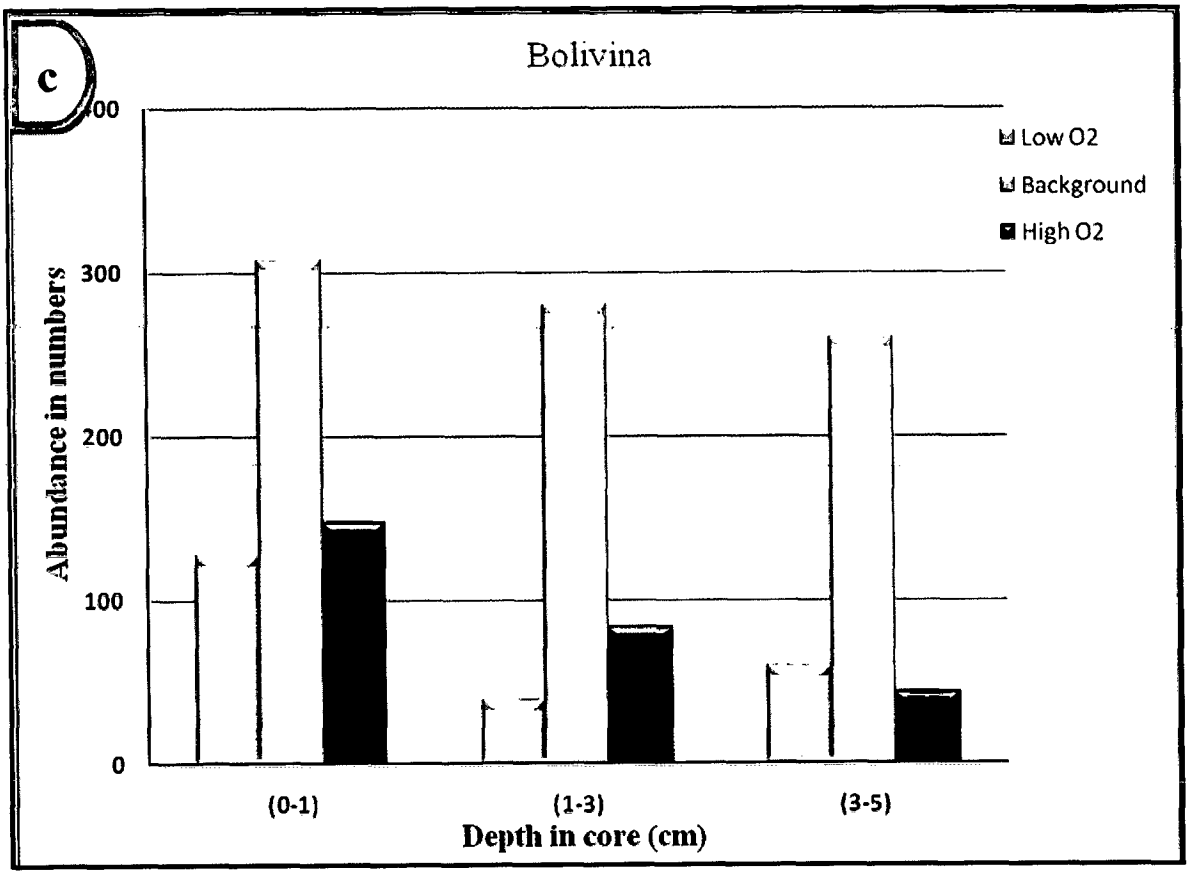
Genera like elongated *Fursenkoina* and rounded *Nonions*, seem to be more adaptive to change in oxygenation. They both are sensitive and move towards zones with better aeration. A contrasting observation can be made about the elongated *Bolivinids* and rounded *Rotalids*. They both show low adaptive abilities, i.e. they tend to die out with any change in oxygenation. Though it is not possible to spell out the behaviour of these foraminifers based on their morphology, we can say that with reduction in oxygen, infaunal species would not only adapt, but would also move to more oxygenated zones i.e. migrate towards the surface. This observation is in agreement with Alve & Bernhard (1995) who have opined that foraminifera select an optimal oxygen regime, regardless of its position with respect to sediment-water interface. However, the *Bolivinid-Rotalid* numbers would drastically fall. This signature if found in subsurface horizons, could be used as an indication of lowering of oxygen in the environments in the past and vice versa. Present observations are in agreement with the TROX model proposed by Jorissen *et al.,* (1995) which suggests that in fully eutrophic environments, it is the critical oxygen level that determines the penetration depth of infauna and in extreme situations where all the oxygen is depleted at the sediment surface deeper sediment layers will be anoxic and all benthic foraminifera will be found exclusively at the sediment-water interface.

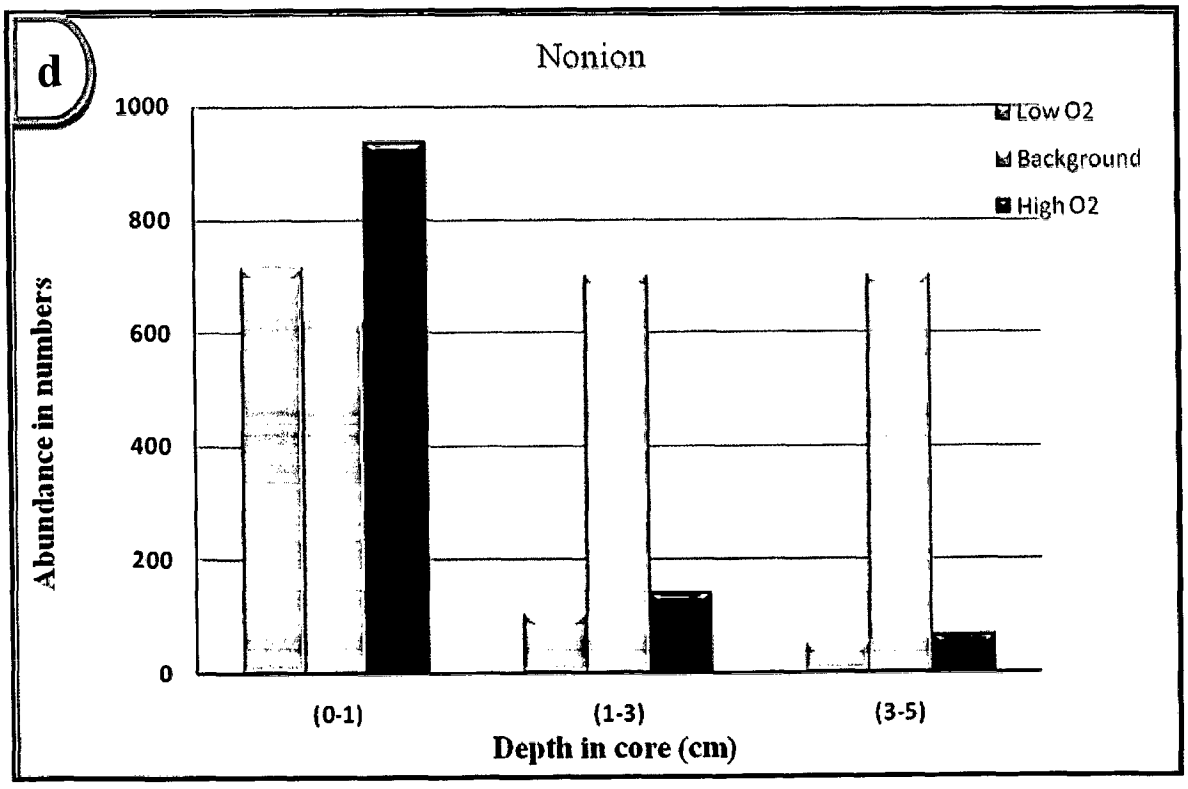
5.4. Conclusions

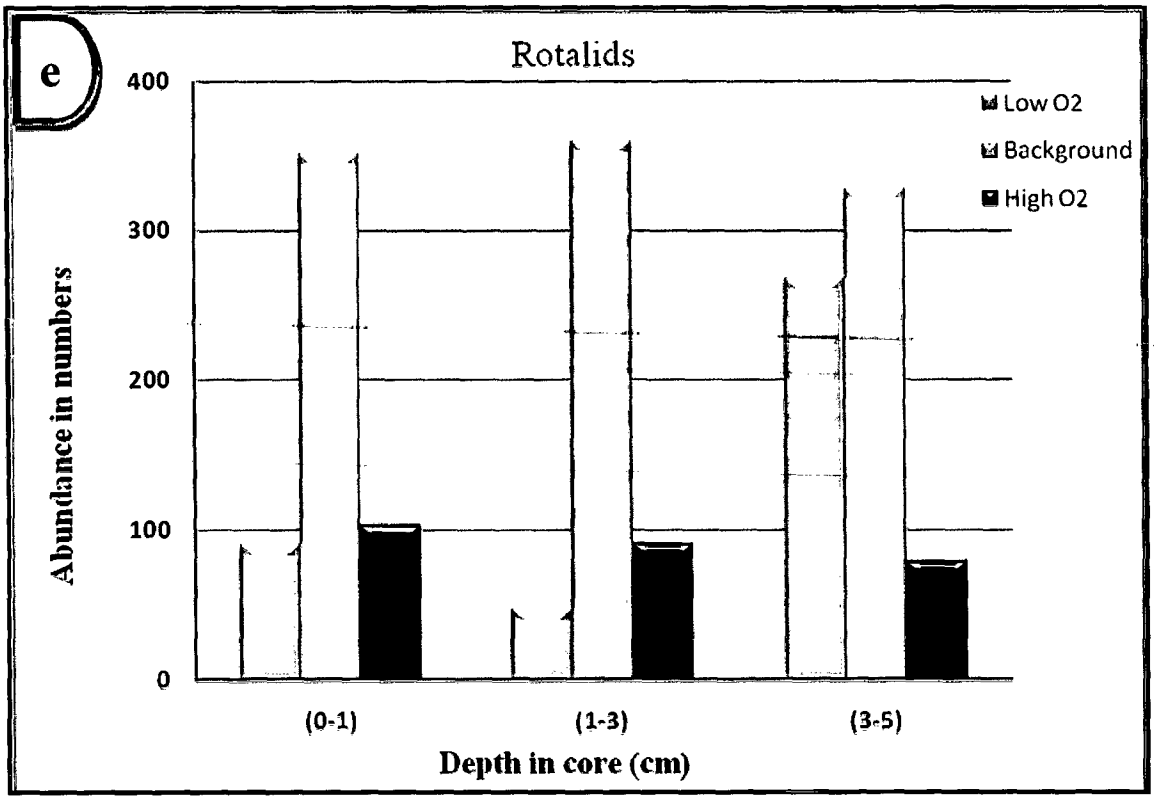
- Any change in natural oxygen conditions affects the foraminiferal abundance.
- Infaunal assemblages are more adaptive to changed oxygen conditions in contrast to epifaunal assemblages, which quickly die out.


a
Total Live Foraminifera
3000
2000
1000
0
Abundance in numbers
Low O2
Background
High O2
(0-1)
(1-3)
(3-5)
Depth in core (cm)


b
Fursenkoina
1000
800
600
400
200
0
Abundance in numbers
Low O2
Background
High O2
(0-1)
(1-3)
(3-5)
Depth in core (cm)


c
Bolivina
00
300
200
100
0
Abundance in numbers
Low O2
Background
High O2
(0-1)
(1-3)
(3-5)
Depth in core (cm)


d
Nonion
1000
800
600
400
200
0
Abundance in numbers
Low O2
Background
High O2
(0-1)
(1-3)
(3-5)
Depth in core (cm)



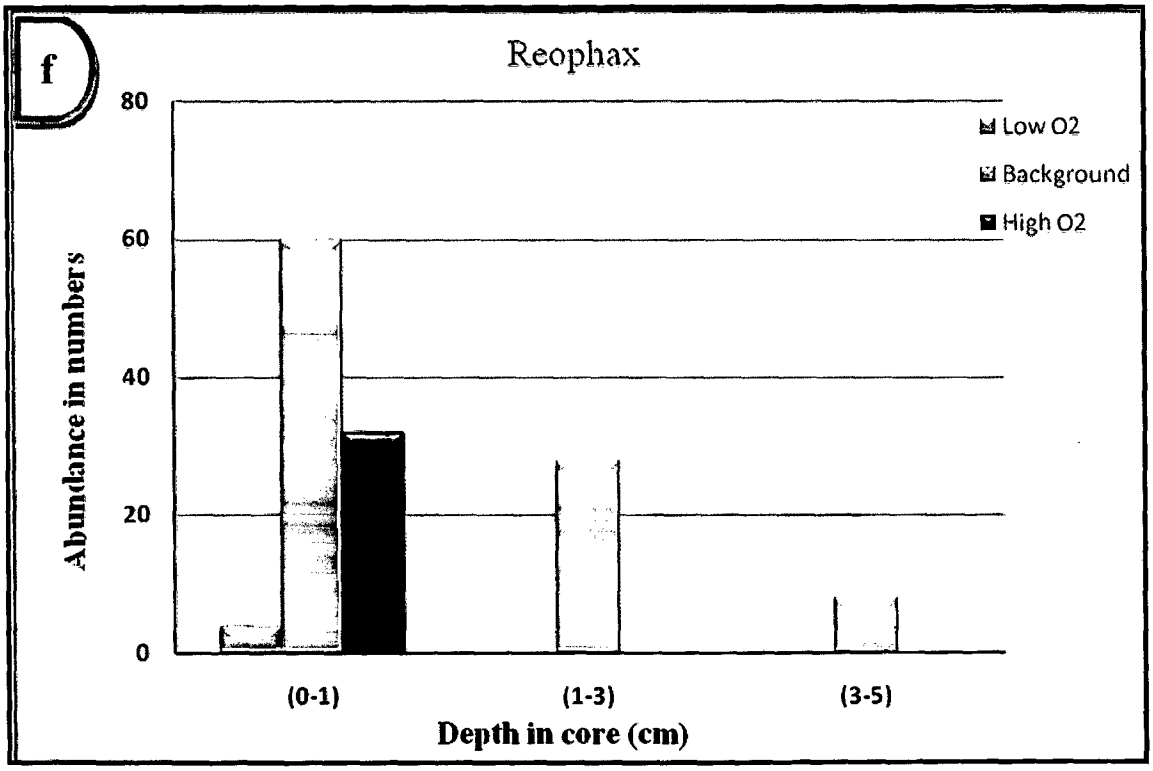


Fig.5.2: Responses of varied foraminiferal groups to oxygen manipulations:
The graphs show variation in abundances of **a)** Total Live Foraminifera; b) Fursenkoina; c) Bolivina; d) Nonion; e) Rotalids; f) Reophax, at different levels in the experimental cores in comparison to the background core

5.5. Significance of the study

The Eastern Arabian Sea is since long known for the existence of its Oxygen Minima Zone (OMZ) (Leutenegger & Hansen, 1979; Sen Gupta *et al.,* 1976; 1980). However, the major concern is about its shrinking or expanding spatial extent due to natural climatic changes as in the past or possibility in the future due to anthropogenic contributions. These can be answered by using proxies developed through such an experimental approach of studying the diverse responses / adaptations of foraminifera to changed oxygen concentrations. Experimental studies like this present attempt only can provide information required to create more accurate databases to be used in other applications of palaeoecology, palaeoceanography, petroleum exploration and pollution studies.

CHAPTER 6

FORAMINIFERAL RESPONSE TO DIFFERENT HEAVY METAL POLLUTANTS

Chapter 6

Foraminiferal response to heavy metal pollutants

"Great things are not done by impulse, but by a series of small things brought together."

- Vincent van Gogh

6.1. Introduction

With the dawn of industrialization, human ways of life changed manifolds. Ways became more consumerist and materialistic, with proliferation of gadgets at its peak. Manufacturing units boomed up. Production increased with demand and thus pollution became rampant. The land on which humans lived, the air breathed were all gradually polluted one after the other. Then, it was water, the cradle of life. Waste, be it solid or liquid; from all the sources, land, water and air finds its abode in the sea. The saga continues. Pollution occurs when concentrations of various chemical or biological constituents exceed a level at which a negative impact on the ecosystem or human health can occur. Pollution results primarily from human activities. More specifically, in the marine context "Pollution of marine environment means the introduction by man, directly or indirectly, of substances or energy into the marine environment, including estuaries, which results or is likely to result in such a deleterious effect as to harm living resources and marine life, hazardous to human health, hindrance to marine activities, including fishing and other legitimate uses of the sea, impairment of quality for use of seawater and reduction of amenities" (http://www.helcom.fi/Convention/en_GB/text). Although marine pollution has a long history, significant international laws to counter it were enacted in the twentieth century. Marine pollution was a concern during several United Nations Conferences on the Law of the Sea beginning in the 1950s. The oceans have long been considered to have limitless capacity to receive and absorb all types of wastes. Since the 1950's many scientists began to warn that this limitless capacity is running out and the very survival of the marine environment was in danger.

Marine pollution can be demarcated into deep sea and coastal pollution. Trade and commerce, merchandise, navigation, fisheries, marine resources etc. have always kept coastal areas bustling with human activities; and so are many reasons for coastal pollution. Moreover, coastal areas also serve as inlets for waste generated on inland regions. Most sources of marine pollution are land based. The pollution often comes

from non-point sources such as agricultural runoff and windblown debris. The sources of contaminants include sewage, urban run-off, industrial wastes, coastal development, and shipping activities. The chemical or biological constituents creating pollution are known as contaminants. More specifically, they can be divided into inorganic contaminants, such as metals; organic contaminants such as pesticides, PCB or petroleum hydrocarbons; Biological contaminants such as coliform bacteria and other pathogens.

Presently the harmful effect of heavy metals as environmental pollutants is widely known and is a matter of concern for the environmentalists because of its lasting effects on biota. Heavy metals are metallic chemical elements that have a relatively high density and are toxic or poisonous at low concentrations. All natural metals occur in sea water in greater or lesser amounts. Some, such as iron, copper, cobalt and zinc are essential in small quantities for the healthy growth of marine organisms. Others, such as mercury, cadmium and lead have no known biological role. All metals are toxic if present in excess but the most important marine contaminants are generally considered to be amongst the non-essential elements. Such toxins can accumulate in the tissues of many species of aquatic life in a process called bioaccumulation. They are also known to accumulate in benthic environments, such as estuaries and bay muds: a geological record of human activities of the last century.

Because they do not degrade or cannot be destroyed, heavy metals are persistent in all parts of the environment. Human activity affects the natural geological and biological redistribution of heavy metals through pollution of the air, water, and soil. The primary anthropogenic sources of heavy metals are point sources such as mines, foundries, smelters, and coal-burning power plants, as well as diffuse sources such as combustion by-products and vehicle emissions. Humans also affect the natural geological and biological redistribution of heavy metals by altering the chemical form of heavy metals released to the environment. Such alterations often affect a heavy metal's toxicity by allowing it to bioaccumulate in the food chain, thereby causing the vivid aftereffects to organisms at different levels of the food chain.

The international community is beginning to recognize the adverse health effects of heavy metals. In 1998, the United Nations proposed the protocol to the convention on long-range trans-boundary air pollution on heavy metals. This protocol is designed to reduce worldwide air emissions of mercury, cadmium and lead, but is yet to be officially adopted (http://www.answers.com/topic/heavy-metals).

This chapter deals with the effect of heavy metals mercury and cadmium on the most popular and widely studied marine protists – foraminifera and the following sections contain some background information on these two heavy metals as pollutants.

6.1.1. Mercury (Hg)

Among the heavy metal pollutants, Mercury (Hg) ranks as the most harmful toxin to the living organisms. In oxygenated marine sediments bacteria may convert the less toxic, inorganic form of the metal to the more toxic, organic form of methyl mercury. This chemical form is relatively mobile in the environment and tends to accumulate in fish. In the most disastrous case of Hg pollution ever reported, over 3000 people suffered from diverse disorders and even died, due to methyl mercury intoxication, which was later known as Minamata disease, from Minamata Bay, Japan. This was reportedly due to the consumption of fish contaminated with heavy concentrations of inorganic mercury discharged into the Minamata bay as waste water by a petrochemical company from 1932–1968. This episode raised a worldwide concern towards the harmful effects of environmental pollution and efforts were initiated towards progress in environment protection measures.

Mercury is an important industrial chemical used, for example, in the chlor-alkali industry and in the manufacture of small batteries. Though the reported amount of dissolved mercury in the marine water sounds negligible, i.e. less than 2 ng/l in open oceans and 50-1000 ng/l of the suspended matter along the continental margins (Mason & Fitzerald, 1996), the concern lies in the fact that it gets biomagnified in the food chain, resulting in higher concentration of this toxic metal in higher level organisms up in the food chain. Through different edible seafood, such as fish and bivalves, Hg finds its way into human beings. Mercury which is a neurotoxin can lead to multifold impacts in humans like memory loss, impaired coordination, vision disturbances, cardiovascular problems, etc. It also affects the thyroid gland, digestive system, liver and skin.

Despite these alarming effects and worldwide concern, as yet no significant effort has been made in India to prevent this heavy metal from reaching the water sources. A news report based on the first global study on mercury by UNEP, states that India may become a hotspot for mercury poisoning owing to the upsurge in gold mining over the last three decades, as reported in "The Times of India" (Mago, 2003). Although there are some reports on Hg pollution, few researchers have studied concentration of mercury in waters off the western coast of India (Singbal *et al.*, 1978; Krishnakumar & Bhat, 1998;

Kaladharan *et al.*, 1999). A minimum of 26 ng/l and a maximum of 187 ng/l of mercury in the regions Off Goa along the west coast of India have been reported (Singbal *et al.*, 1978). Although, based on field studies, a few attempts were made to document benthic foraminiferal response to trace metal pollution along the Indian coasts (Naidu *et al.*, 1985; Sivakumar *et al.*, 2008), harmful effects of Hg were not discussed.

6.1.2. Cadmium (Cd)

Cadmium (Cd), like mercury, is an industrially important metal which is used directly in plastics and electroplating and is also found in applications associated with zinc and phosphorus. Although cadmium has no known biological role it is taken up by marine phytoplankton; apparently by the same mechanism employed for the uptake of the essential nutrient element phosphorus. It has been demonstrated to stimulate phytoplankton growth and photosynthesis up to surprisingly high levels. Even in quite heavily contaminated marine systems cadmium is very unlikely to cause acute toxic effects to marine animals but there have been at least two instances where cadmium pollution has apparently been responsible for adverse effects to humans. The best known example of this was the outbreak of itai-itai disease in Japan which affected a village on the Jintsu river in Japan. The use of irrigation water, contaminated with cadmium from a zinc processing plant, in paddy fields was originally identified as the cause of the problem but other factors such as malnutrition and vitamin deficiency have also been cited as major contributory parameters. *Itai-itai* disease is characterized by brittle bones and considerable pain (the name means 'ouch-ouch') and the symptoms can be alleviated by the administration of large doses of vitamin D. The other well known instance of cadmium poisoning was caused by the consumption of contaminated Tasmanian oysters which led to nausea and vomiting in the victims.

Various chemical, physical, physiological as well as biological methods have been formulated and adopted to detect marine pollution. Conventional biological methods of pollution detection have included the estimation of levels of pollutants in both micro and macro marine organisms. Marine organisms like bivalves, fishes etc have the potential to sequester toxic pollutants in their tissues and gradually, these toxicants get biomagnified as they traverse the higher trophic levels. These organisms can detect and incorporate pollution signatures of a very short time span in them, and cannot be representative of the past, either remote or near.

Marine pollution is manifest when organisms of the higher trophic levels are affected, so a base level detection of pollutants is not achieved. Many species of marine microorganisms like bacteria have been extensively used to detect marine pollution on a base level but since bacteria lack fossilization potential they are again of limited use in recording the marine environment of the distant or the near past.

An effective tool to curb the problem of time series pollution detection is by using foraminifera as proxy for pollution detection. Foraminifera are microscopic, unicellular, almost exclusively marine organisms with a hard test. Foraminifers occupy the base level of the food chain. They are abundantly distributed vertically as well as geographically. Being microscopic they have a very short life span. They are highly sensitive to the changes, which occur in their ambience. These changes get incorporated in their test. The test has fossilization potential and the changes taking place in its environment gets preserved in their test even after the death of the organism. Thus foraminifer's fossils from a given region serve as an effective tool to detect the quality of the marine environment (including pollution) of that region at that given time as well as of the past.

6.1.3. Benthic foraminifera in marine pollution studies

Benthic foraminiferal distributions in polluted areas have been investigated for more than four decades and several workers have pointed out that these organisms provide one of the most sensitive markers available for inferring the deterioration of marginal marine environments (Alve, 1995). After pioneering work on the effects of pollution on benthic foraminifera by Zalesny (1959), pollution studies using benthic foraminifera as proxy indicators were started by Resig (1960) and Watkins (1961). Since then numerous workers focused on the effects of various pollutants on biota from different marginal environments. Initially, most of such studies dealt with the effects of organic waste contamination (Bandy *et al.*, 1964a, 1964b; Seiglie, 1968; Schafer, 1973, Schafer *et al.*, 1975), and little attention was paid to understanding the response of foraminifera to heavy metal pollution (Alve, 1991; Nigam *et al.*, 2006b).

The majority of the above-mentioned studies reported variation in species abundance in the polluted areas. Also, there were reports of deformed tests from the polluted sites. Although morphological deformities in fossil and recent foraminiferal tests from polluted sites have been noticed since the 19th century (Carpenter, 1856; McCrone & Schafer, 1966; Setty & Nigam, 1984; Naidu *et al.*, 1985; Bhalla & Nigam, 1986; Stouff *et al.*,

1999a; Yanko *et al.*, 1999; Naidu *et al.*, 2000; Debenay *et al.*, 2001; Frontalini & Coccioni, 2008; Romano *et al.*, 2008), the interpretation of these results was doubted when similar deformities were reported from areas subjected to natural stress like abnormal salinity (Freudenthal *et al.*, 1963; Hofker, 1971; Brasier, 1975; Reiss & Hottinger, 1984; Nigam *et al.*, 2006a), change in nutrient levels (Murray 1963) and rapid change in environmental conditions (Scott & Medioli, 1980). Boltovskoy & Wright (1976) opined that abnormalities in foraminiferal test can also be due to mechanical damage. If such ambiguities are to be solved, there should be some standard results in controlled conditions wherein the effect of different parameters can be studied in different combinations and with which the field results can be compared.

Laboratory culture experiments provide such continuous and accurate observations on the foraminiferal response under controlled conditions. Here a single parameter can be altered, keeping the rest constant in order to observe the foraminiferal response to variation in a particular parameter. In this way, foraminiferal response to specific parameters can be characterized, adding credibility to the field based observations (Nigam *et al.*, 1996a; 1996b; Saraswat *et al.*, 2004; Filipsson, 2008).

In the light of the discussion above, the effect of varying concentrations of heavy metals mercury and cadmium on benthic foraminiferal species *Rosalina leei* and *Pararotalia nipponica* respectively was studied through laboratory experiments and the same has been described in the following segments of this chapter.

Samples containing live foraminifera for the experiments were collected from the shallow-water areas off Goa, the central west coast of India near to National Institute of Oceanography, Goa as per the methods described in chapter 3.

6.2. Gradual increase in mercury concentrations: Effect on benthic foraminifera *Rosalina leei*

6.2.1. Experimental setup

The juvenile specimens of *Rosalina leei* were subjected to different concentrations of mercury prepared by dissolving water soluble mercuric chloride in seawater. One set was maintained without any mercury and served as control. A total ten sets of media were prepared with different mercury concentrations ranging from 20 ng/l to 180 ng/l at 20 ng/l intervals. In order to avoid a sudden shock to the organisms, the Hg concentration was increased gradually at every alternate day keeping one set at each concentration (Fig. 6.1). Two specimens each were maintained in all mercury

concentrations prepared. To find out the maximum Hg tolerance limit of this species, the specimens kept at 180 ng/l were later subjected to mercury concentrations up to 260 ng/l. Measurements such as the maximum diameter and number of chambers were taken at every alternate day. Additional observations like the pseudopodial activity, shape and orientation of newly added chambers were also made. Other parameters including salinity (35 ‰), temperature (Room Temperature), feed (*Navicula*) and light (12 hr light- 12 hr dark) were maintained uniform throughout the experiment

6.2.2. Results

The specimens showed extensive pseudopodial activity at the onset of the experiment. With gradual increase in the mercury concentration, the pseudopodial activity started declining and almost ceased in 4-5 days in case of specimens subjected to 100 ng/l. The specimens were still alive and were accumulating food near the last chamber very slowly. Similar trend was observed in the growth rate also. The maximum growth in diameter attained by the specimens showed an inverse relation with the mercury concentration in the medium (Fig. 6.2), i.e. specimens kept at higher Hg concentration attained comparatively less growth. Growth nearly ceased after 40 days at all concentrations, but the specimens continued to live. The specimens were alive even when the concentration was increased to 260 ng/l. The maximum size attained by the experimental specimens was less than the normal size of the field specimens. Most importantly, it was found that chambers added to the specimens kept at higher concentrations of mercury, were abnormally large and with unusual orientation.

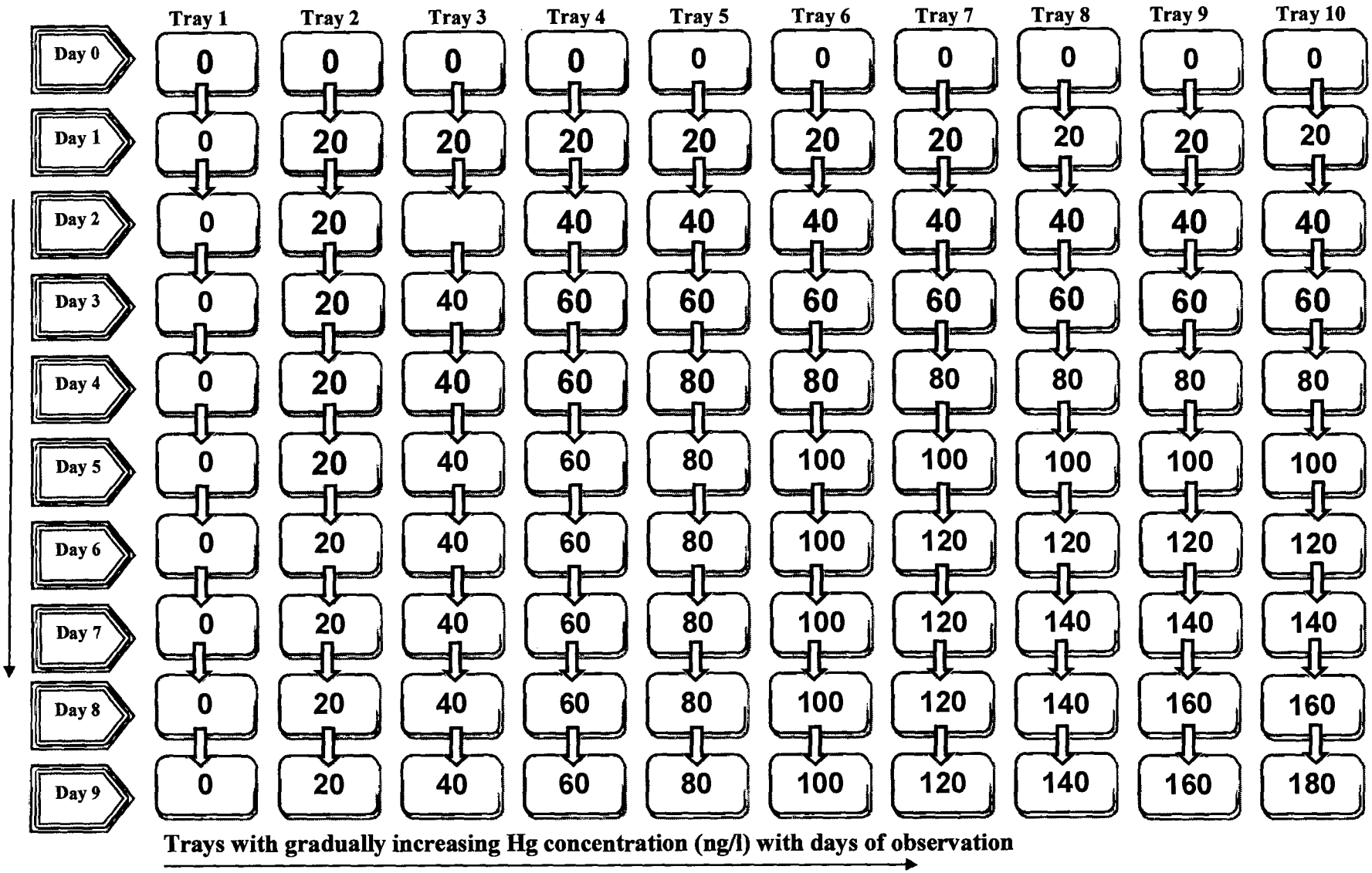


Fig. 6.1: Set up of Experiment to decipher the effect of gradual additions of mercury on *Rosalina leei*:
Days = days of observation; **Trays** (represented by the rectangles) are labeled with their respective concentrations of mercury (in ng/l) on each day of observation

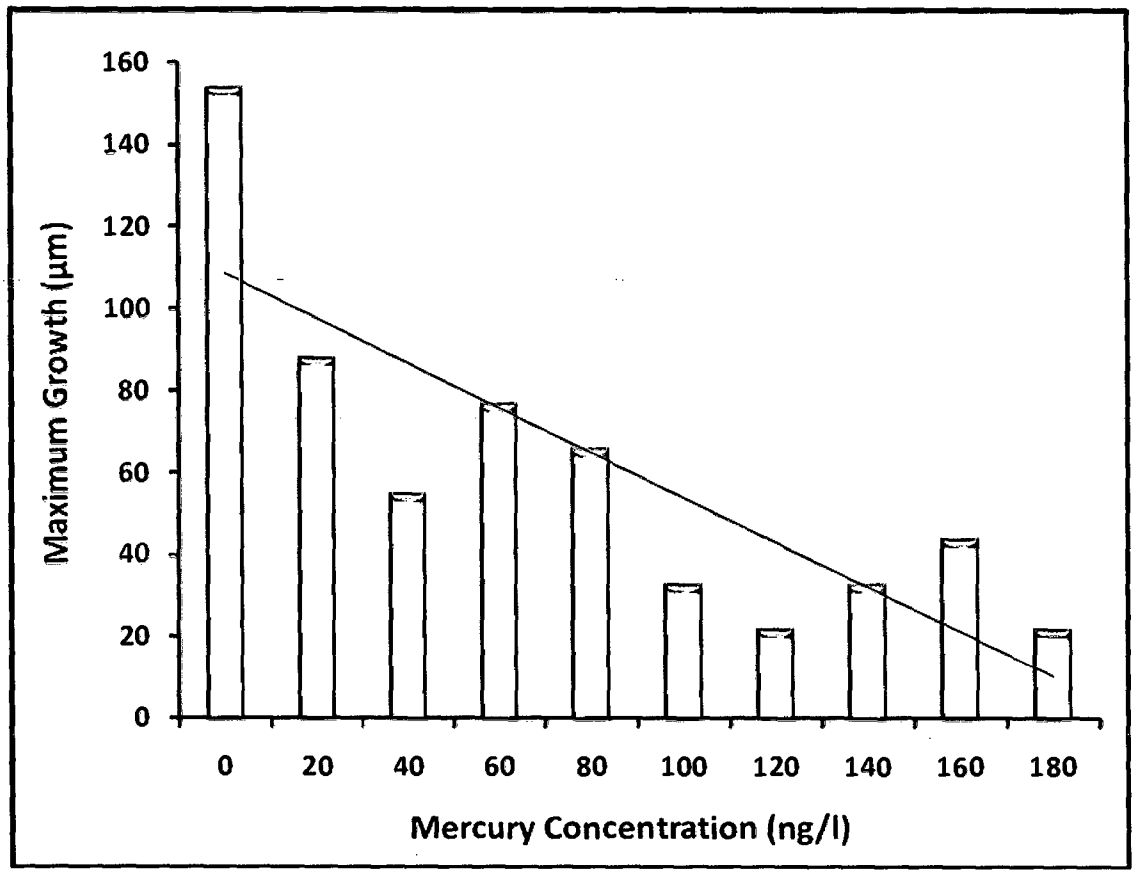


Fig. 6.2: The maximum growth attained at different concentrations of mercury in the experiment in which the amount of Hg was increased gradually

6.3. Sudden increase in mercury concentrations: Effect on benthic foraminifera *Rosalina leei*

6.3.1. Experimental Setup

Juvenile specimens of *Rosalina leei* were subjected to different concentrations of mercury prepared by dissolving water soluble mercuric chloride in seawater. One set was maintained without any mercury and served as control. A total twelve sets of media were prepared with different mercury concentrations from 25 ng/l to 300 ng/l at 25 ng/l intervals. In order to see the response of benthic foraminifera to sudden stress, the organisms were directly exposed to the respective concentrations (Fig. 6.3). Five specimens each were maintained in all mercury concentrations prepared. Other parameters such as salinity (35 ‰), temperature (25 °C), feed (*Navicula*) and light (12 hr light - 12 hr dark) were maintained uniform throughout the experiment. The specimens were observed every third day and their responses were observed.

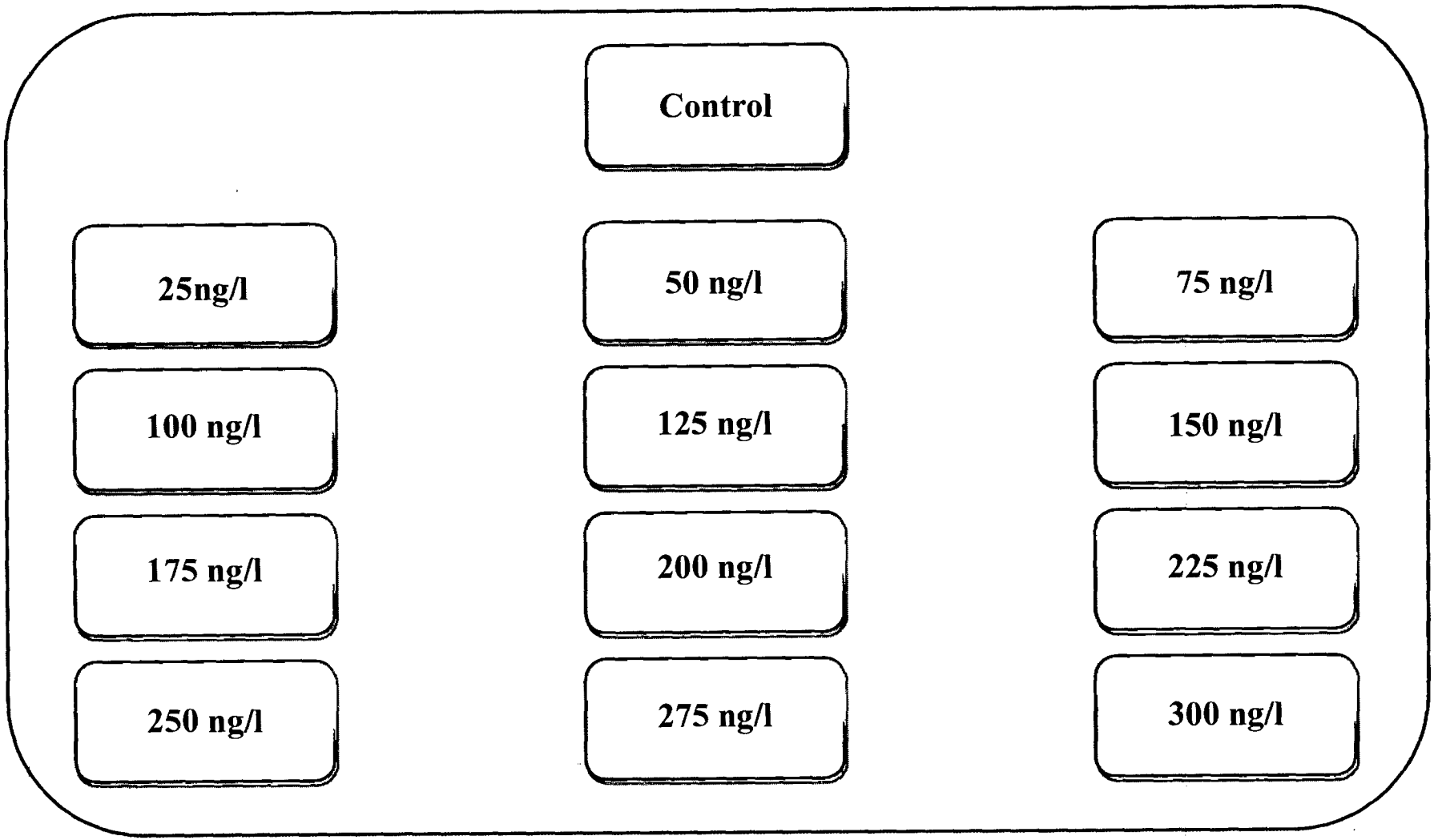


Fig. 6.3: Set up of Experiment to decipher the effect of sudden additions of mercury on *Rosalina leei*:
Trays (represented by rectangles) are labeled with their respective concentrations of mercury (ng/l) during the experiment

6.3.2. Results

For the first 40 days of the experiment, the specimens did not show any visible change in morphology, but later morphological abnormalities started to appear. The abnormalities included larger than normal size of the last chambers, and abnormal orientation and shape of the new chambers (Plate 6.1). When the percentage of specimens deformed at each concentration is plotted, it showed that up to 150 ng/l, 20% - 75% of the specimens were deformed, whereas at Hg concentrations above 150 ng/l and up to 275 ng/l, all the specimens showed deformation (Fig. 6.4). Specimens kept at 300 ng/l died after an exposure of 19 days to the mercury concentration. None of the control specimens showed any morphological deformation throughout the experiment.

Apart from the morphological deformation irregular reproduction was also noticed in the experimental specimens (Fig. 6.5). As compared to the control specimens, a higher number of specimens subjected to Hg concentration reproduced.

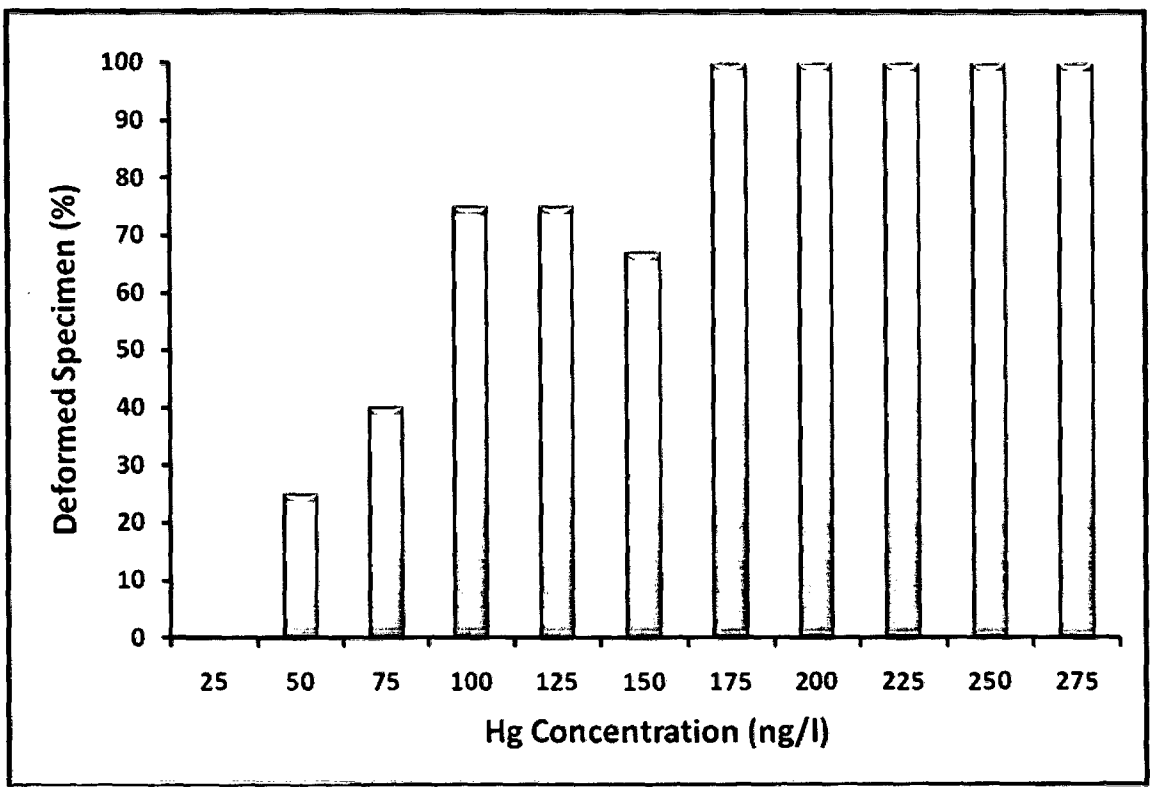


Fig. 6.4: Percentage of deformed specimens at different mercury concentrations in the second set of experiment where Hg was suddenly introduced.

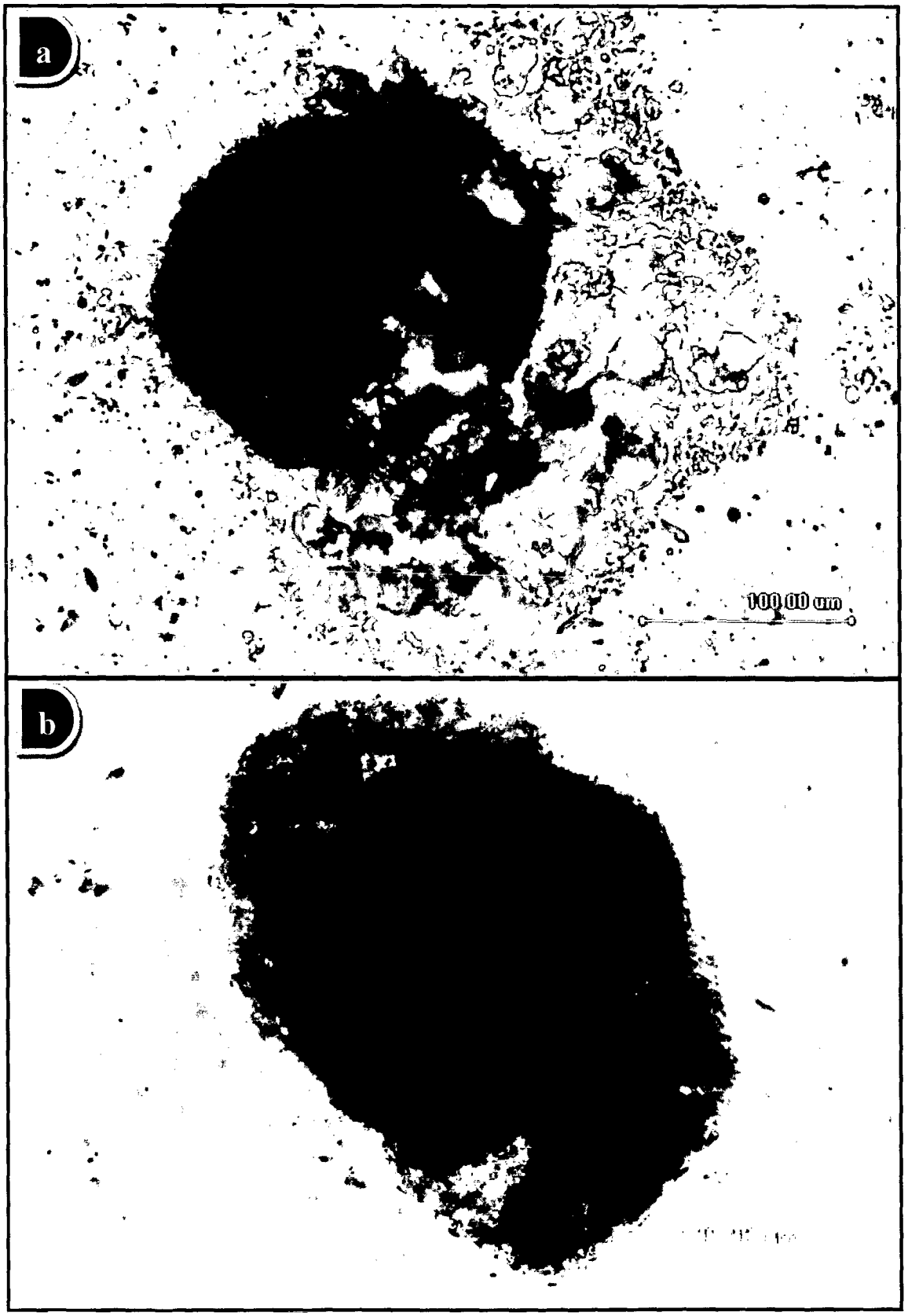


Fig. 6.5: (a) Reproduction in specimens kept in media without Hg (b) Reproduction in specimens kept in higher Hg concentrations

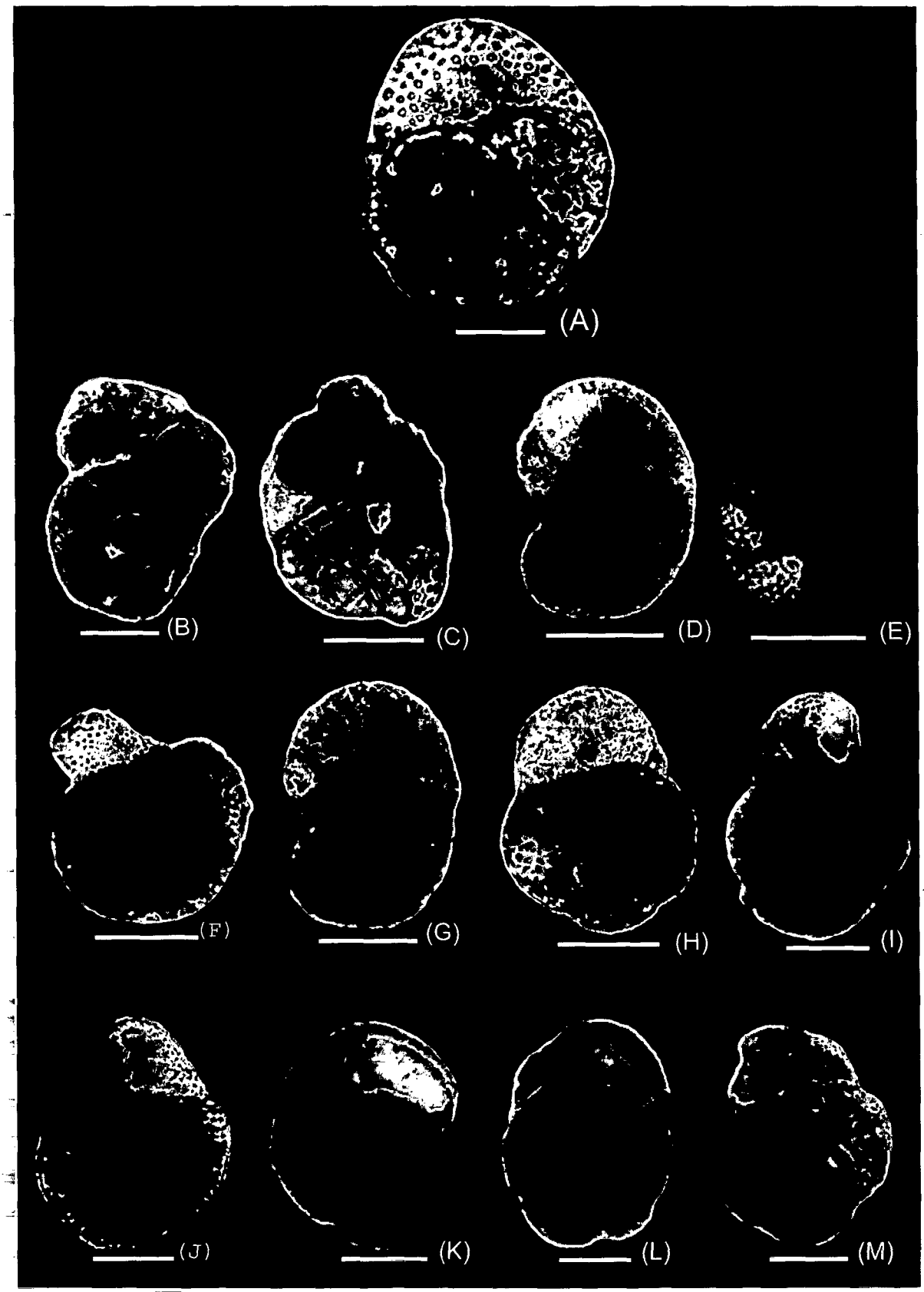


Plate 6.1: Different types of abnormalities (B-M) reported in specimens subjected to higher concentrations of Hg. Fig A shows a normal specimen. Scale bar = 100 μm

At lower concentrations (25 ng/l, 50 ng/l), none of the specimens reproduced throughout the experiment, but at 75 ng/l to 225 ng/l, reproduction was reported at all seven concentrations. In specimens subjected to 75 ng/l, 100 ng/l, 175 ng/l, 200 ng/l and 225 ng/l, the number of juveniles produced after reproduction varied from 6-12 but in case of specimens subjected to 125 ng/l and 150 ng/l Hg concentration, the number of juveniles were 23 and 20 respectively. The juveniles died within a day or two in all the cases. At still higher concentrations 250 ng/l and 275 ng/l, juveniles did not come out of the mother specimen at all. On the contrary, reproduction in the control specimens produced a minimum of 30-35 juveniles per mother specimen.

6.4. Gradual increase in Cadmium concentrations: Effect on benthic foraminifera *Pararotalia nipponica*

6.4.1. Experimental Setup

For the experiment, cadmium chloride monohydrate (atomic weight 201.32) was used to prepare a stock solution of 500 µg/l concentration by dissolving $CdCl_2.H_2O$ in filtered seawater ($CdCl_2$ being the soluble form of cadmium). The range of cadmium concentration was decided on the basis of previous literature. According to Krishnakumar *et al.*, (2004), the mean cadmium concentration in seawater samples collected from west coast of India was 2.95 µg/l, However, at a few stations the Cd Concentration reached even up to 8.15 µg/l. Based on these records it was decided to subject the foraminifers to Cd concentration range from 2 µg/l to 14 µg/l with an interval of 2 µg/l. In order to avoid a sudden shock to the foraminifers the cadmium was added gradually in to the media starting from 2 µg/l till the desirable concentration is reached as per the setup (Fig. 6.6)

A set of four specimens was maintained at each concentration with replicates. Before the onset of the experiment, all the specimens were photographed and measured for the maximum diameter and number of chambers using the inverted Microscope (software ACT-2U). Prior to addition of every higher Cd concentration, the specimens were photographed and its maximum diameter was measured in order to observe and record the changes brought about by Cd administration. After adding the Cd solution of highest concentration (14 µg/l), the concentration was further increased in order to understand the tolerance *of P. nipponica* to cadmium.

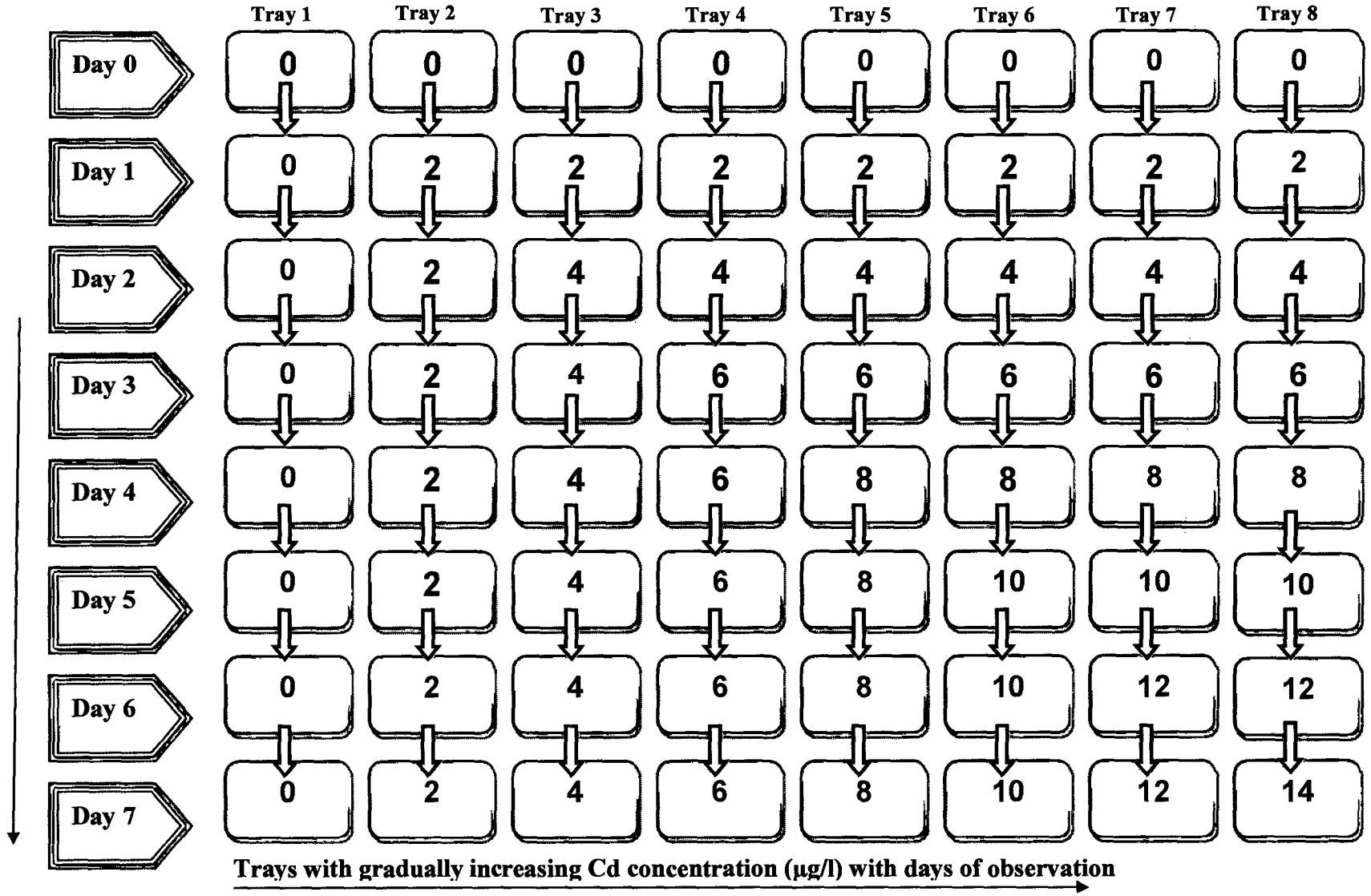


Fig. 6.6: Set up of Experiment to decipher the effect of gradual additions of cadmium on *P.nipponica*

Days = days of observation; **Trays** (represented by the rectangles) are labeled with their respective concentrations of cadmium (in µg/l) on each day of observation

The set-up of the experiment is explained in figure 6.6, where the rectangles indicate the experimental trays where cadmium concentrations were gradually increased from 2 μg/l to 14 μg/l. Specimens were monitored throughout the experiment for their response to cadmium in terms of growth and morphological changes.

6.4.2. Results

At the onset of the experiment all specimens were healthy and showing extensive pseudopodial activity and actively feeding up on the diatoms. With the progressive addition of cadmium in to the media the specimens other than the control specimens showed reduction in their pseudopodial activity and developed visibly abnormal chambers. This phenomenon was common at all cadmium concentrations right from the lowest 2 μg/l to the highest 14 μg/l; only the degree of deformity in the specimens varied. The specimens at higher concentrations showed severe deformities compared to the ones kept in lower concentrations (plate 6.2).

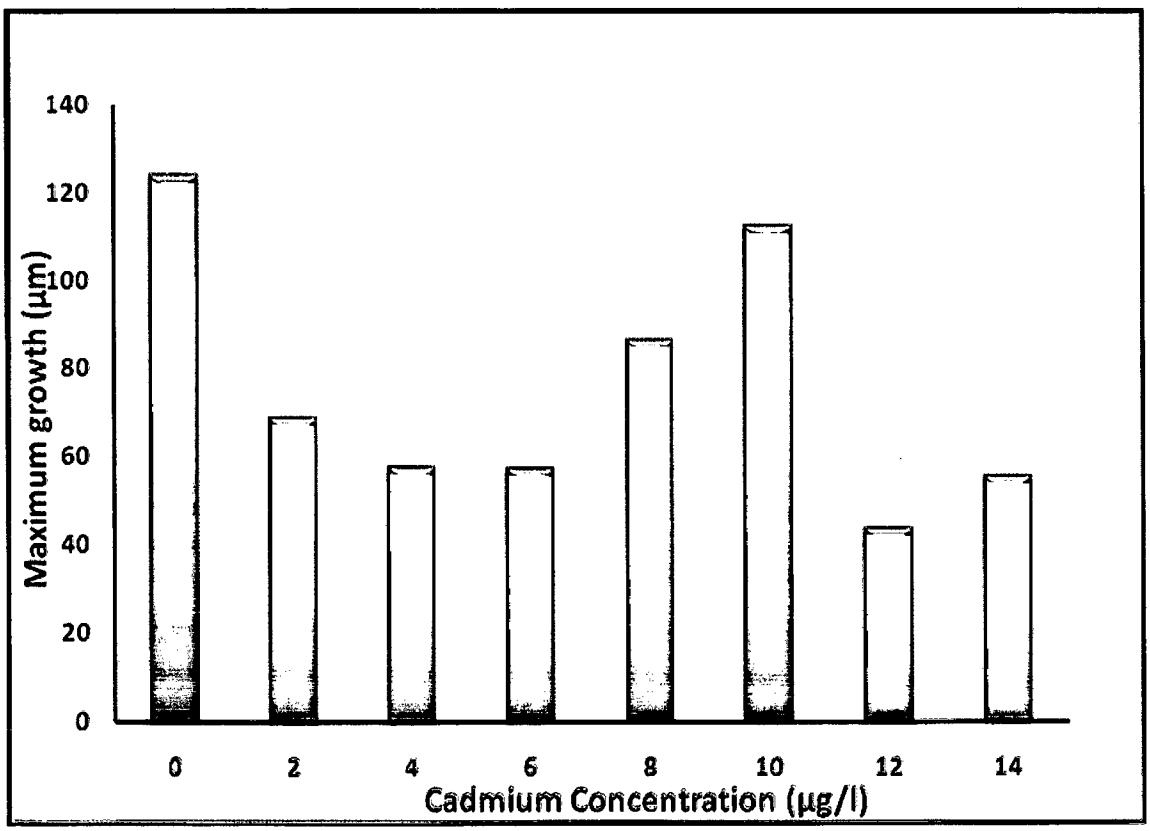


Fig. 6.7: The maximum growth attained at different concentrations of cadmium in the experiment in which the amount of Cd was increased gradually

An interesting observation regarding the pattern of deformation in the specimens grown at high Cd concentration are the changes in coiling direction. The abnormal chambers formed during the experiments were added in to a different plane as evident from the comparison between the control specimen and experimental specimens given in plate 6.2.

Due to the same reason, the growth could not be measured effectively and the changes in the maximum diameter of the specimens during the experiment do not show any trend with the increase in concentration of cadmium in to the media (Fig. 6.7). But compared to the controlled specimens, all the experimental specimens show less growth. While the control specimens grown in field conditions without any cadmium showed an average growth of 124.31μm, all the experimental specimens subjected to various cadmium concentrations from 2μg/l to 14 μg/l showed growth varying from 112.69 μm to 44.02 μm. All the experimental specimens were alive in all the concentrations during the span of the experiment. The concentration of cadmium was further increased beyond 14 μg/l to 16 μg/l in the final set; but the specimens continued to survive though remained inert.

6.5. Discussion

Heavy metals certainly have deleterious effect on benthic organisms in general (Aschan & Skullerud, 1990) and foraminifera in particular (Yanko *et al.*, 1998). Much has been discussed on the controversies surrounding the use of morphological deformities of foraminiferal tests as a tool to identify the presence of various pollutants. The concern is to characterize and differentiate between the foraminiferal response to natural stress (such as temperature, salinity changes) from that to anthropogenic pollutants through a series of field and laboratory culture studies (Nigam *et al.*, 1996a, b; Yanko *et al.*, 1998; Samir, 2000; Scott *et al.*, 2001; Geslin *et al.*, 2002, Saraswat *et al.*, 2004; Romano *et al.*, 2008). Working towards this objective, laboratory culture experiments were attempted to study the response of the benthic foraminifer -

- *Rosalina leei* to varying (gradual as well as sudden changes) concentrations of heavy metal mercury
- *Pararotalia nipponica* to varying concentrations of heavy metal cadmium; which are discussed separate in the coming sections for effective and clear conveying of the ideas.

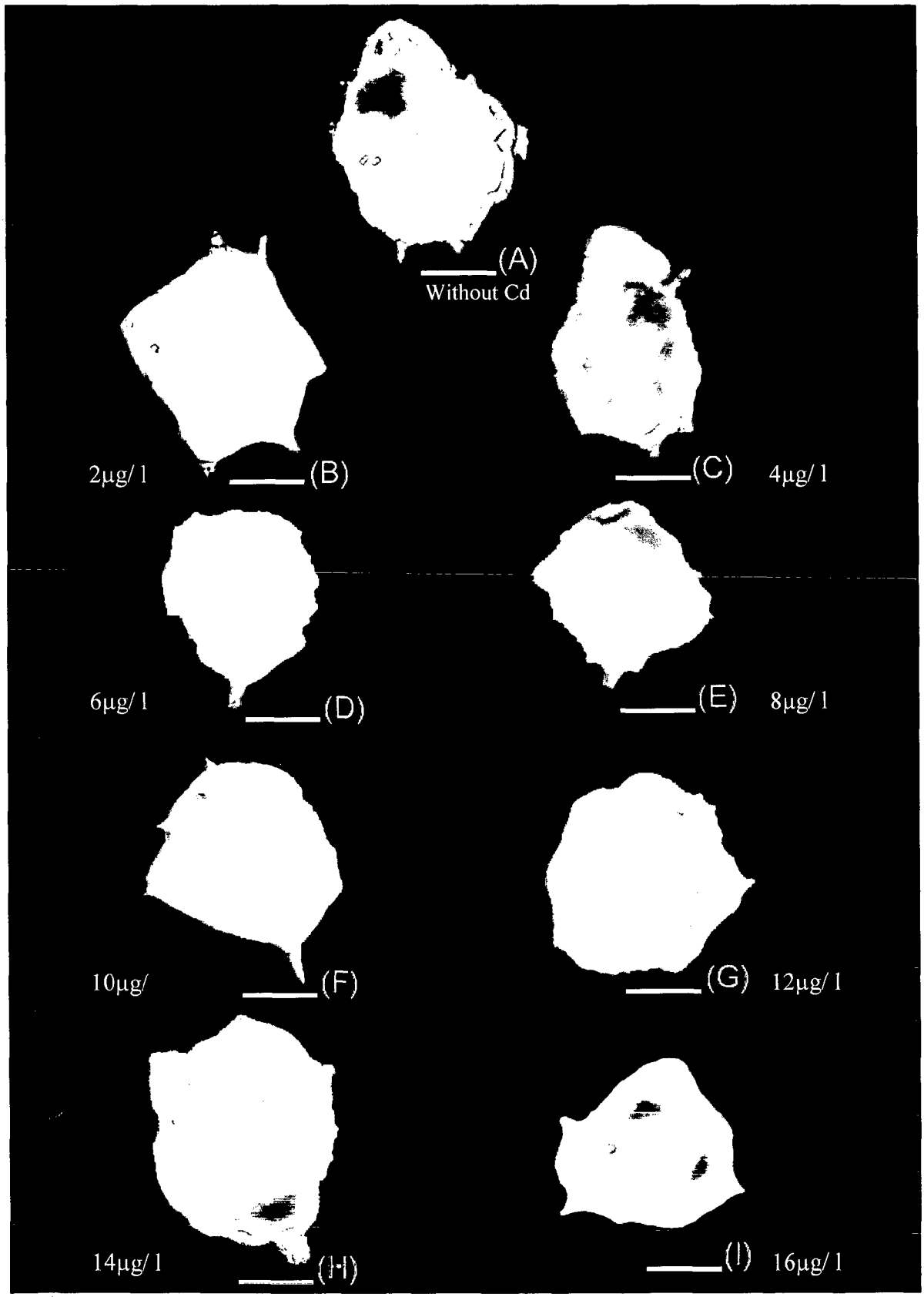


Plate 6.2: Different types of abnormalities (B-M) reported in specimens subjected to different concentrations of cadmium. Fig A shows a normal specimen. Scale bar = 100 µm

6.5.1. Effect of heavy metal mercury on *Rosalina leei*

A distinct difference is noted in the response of *R. leei* to gradual and sudden increase in Hg concentration. The results of the first experiment show that gradual exposure to mercury concentrations affected the normal growth of the specimens and the growth was inversely proportional to Hg concentration. Although deformation was reported, but only in specimens subjected to higher Hg concentrations and the number of abnormal specimens was very low. Contrastingly, in specimens subjected to sudden stress, the percentage of deformed specimens was very high (75-100% of the total specimens per conc.). Abnormalities included change in the plane of addition of new chambers, leading to no net increase in the maximum diameter of the specimen. As, in order to measure incremental and overall growth, size in terms of maximum diameter of the individual specimen has to be measured, such measurements were not possible in highly deformed specimens under sudden exposure to different Hg concentrations. Therefore, percentage of deformed specimens was plotted which shows a good correlation with different Hg concentrations. Although the number of deformed specimens was more while being subjected to sudden stress, the test abnormalities were similar as that in the earlier experiment, i.e. abnormal size, shape and orientation of newly added chambers. A significant effect on growth but low number of deformities in specimens subjected to gradually added Hg, while a large number of deformities in specimens subjected to sudden Hg exposure, probably indicate the adaptive capability of *R. leei*. In the experiment wherein Hg was added gradually, probably no severe damage was done to the normal physiology of the *R. leei* specimens and enough time was available to adapt as per the changed condition by way of lowering the growth rate. Whereas, in specimens directly subjected to different concentrations of Hg, irreparable damage to the soft tissue probably leads to increased abnormalities.

The decline in the pseudopodial activity at the early stages of both the experiments indicates that the cytoplasm is immediately affected by the presence of Hg in the medium. Similarly the inverse relationship between Hg concentration and growth indicates that the presence of heavy metal in the medium inhibits the normal metabolic activity of the organism. The decreased metabolic activity slows down the growth, as a result of which, the specimens subjected to different mercury concentrations attain lower average size as compared to the field specimens. This explains the occurrence of stunted specimens at polluted sites (Yanko *et al.*, 1994, 1998; Samir & El-Din, 2001).

The findings confirm the views of Boltovskoy & Wright (1976) who noted that 'the presence, absence, disequilibria or inter-relations of some of the trace elements in individual organisms can retard or stop normal growth, can provoke abnormal development (monstrosities) and can even induce death'.

The addition of abnormal chambers at higher mercury concentrations and morphological abnormalities after prolonged exposure to the pollutant, explains the occurrence of abnormal tests in areas subjected to various pollutants as reported for decades by a number of previous workers (Watkins, 1961; Lidz, 1965; Seiglie, 1971, 1975; Bhalla & Nigam, 1986; Sharifi *et al.*, 1991; Alve, 1991a; Yanko *et al.*, 1998; Geslin *et al.*, 1998; Samir, 2000; Debenay *et al.*, 2001; Samir & El din, 2001; Scott *et al.*, 2005, Bergin *et al.*, 2006, Frontalini & Coccioni, 2008). Since, during the experiment, all the parameters other than the Hg concentration were kept constant, the response can be attributed to the effect of mercury.

Increased instances of stress induced reproduction were one of the main effects of sudden addition of Hg, as seen in second experiment. Specimens subjected to all but 25 ng/l and 50 ng/l Hg concentrations, reproduced under sudden addition of Hg into the medium. Additionally, reproduction was significantly different in specimens subjected to sudden Hg stress, than that in control sets. The specimens subjected to sudden addition of Hg, produced less number of juveniles and they could not survive for more than 2 days in the medium. Additionally, in specimens subjected to the highest Hg concentration, juveniles could not come out and died within the mother cell. Although reproduction was also noted in earlier experiment (gradual addition of Hg), but only a few specimens subjected to comparatively high Hg concentration reproduced.

Reproduction in foraminifera is controlled by the environmental factors. The optimum range of environmental conditions required for the successful reproduction in foraminifera is very narrow compared to the optimum range for their survival (Murray 1963). A deviation from the optimum conditions cause variations in the reproductive behaviour in foraminifera. Although few in number, there are previous reports addressing this particular aspect of the ecological preferences of foraminifera. Myers (1935b) and Bradshaw (1957) reported that lower temperature leads to a delayed reproduction in foraminifers. In a subsequent study Bradshaw (1961) reported that higher temperatures lead to quick reproduction in foraminifera. Ross (1977) maintained that reproduction is linked to the seasonal changes in food. Hemleben and Kitazato (1995)

reported that the culture maintained without food survived for longer duration but reproduced less than the ones maintained under continuous food supply. This shows the influence of food on the normal reproduction in foraminifers. Like the natural stresses, anthropogenic pollutants also control the reproduction in foraminifers.

Moodley *et al.* (1998a) reported that sulphidic conditions resulted in total lack of reproduction in foraminifera. According to Cadre and Debenay (2006), copper contamination resulted in delayed reproduction, whereas in another recent study by Ernst *et al.* (2006), few species reproduced quickly under induced oil pollution. In the light of these previous studies it can be concluded that foraminiferal response varies with stresses. In the present study, It was observed that number of reproduction in specimens subjected to the increased concentration of Hg is more as compared to the control specimens. The observed differences in reproduction and the number of juveniles produced, in specimens maintained with and without Hg, conclusively show that heavy metal pollutants affect the normal growth as well as reproduction in *Rosalina leei.* The death of the juveniles soon after the reproduction, in the specimens kept at high Hg concentration, may be because of the fact that the juveniles from this abnormal reproduction were probably not healthy to cope with the sudden stress in the form of high Hg content. In the light of the findings, this study reinforces the views expressed by Alve (1991b) that "in extreme cases of heavy metal pollution, the organism devotes it energy to protect itself. As a result, such an individual has little ability left for protein synthesis. This inhibits the energy budget, reproduction cycle, and also harms the cytoskeleton". This may also be a probable explanation for the reduced number or gradual absence of some species of benthic foraminifera from the areas subjected to pollution. As there are not many previous reports in this line, the present attempt is significant in characterizing the foraminiferal response to different heavy metal pollutants.

6.5.2. Effect of heavy metal cadmium on *Pararotalia nipponica*

The decline in the pseudopodial activity in the experimental specimens other than the control specimens suggests that the cytoplasm is immediately affected by the addition of cadmium in to the media and is explained on the basis of the studies by Bresler and Yanko (1995), which says that there is significant biological influence of heavy metals on foraminiferal cytoplasm.

The lesser growth attained by the specimens grown in media with cadmium compared to the specimens grown without cadmium indicates that the presence of cadmium in the medium inhibits the normal metabolic activity of the organism thereby slowing down the growth, resulting in stunted specimens. This explains the occurrence of stunted specimens at polluted sites (Yanko *et al.*, 1994, 1998; Samir & El-Din, 2001).

The addition of abnormal chambers during the course of the experiment in the specimens subjected to cadmium concentrations suggest that the heavy metal cadmium is adversely affecting the *P. nipponica* specimens and since all parameters other than the cadmium concentrations were maintained constant, the response can be attributed to the presence of cadmium in the media. The development of deformities in all the specimens subjected to different Cd concentrations right from the lowest 2 µg/l) to the highest (14 µg/l) indicates the toxic effect of the heavy metal cadmium even at lower concentrations. The change in the coiling plane due to the addition of abnormal chambers to a plane different than the normal specimens is a peculiar observation made from the experiment. The reduction in growth by the cadmium addition and the development of abnormalities are in a way similar to the effect of mercury and once again confirm the views of Boltovskoy & Wright (1976) who noted that 'the presence, absence, disequilibria or inter-relations of some of the trace elements in individual organisms can retard or stop normal growth, can provoke abnormal development (monstrosities) and can even induce death' and once again explains the occurrence of abnormal tests in areas subjected to various pollutants as reported for decades by a number of previous workers (Watkins, 1961; Lidz, 1965; Seiglie, 1971, 1975; Bhalla and Nigam, 1986; Sharifi *et al.*, 1991; Alve, 1991b; Yanko *et al.*, 1998; Geslin *et al.*, 1998; Samir, 2000; Debenay *et al.*, 2001; Samir & El din, 2001; Scott *et al.*, 2005, Bergin *et al.*, 2006, Frontalini and Coccioni, 2008) as explained in the section above.

6.6. Conclusions

6.6.1. Effect of heavy metal mercury on *Rosalina leei*

- The response of benthic foraminifera *Rosalina leei* is different and distinct to gradual and sudden stress conditions.
- On gradual increase in the mercury concentrations, specimens showed lesser growth with increasing Hg concentration.

- Sudden addition of mercury increased abnormal reproduction which is significantly different from the normal pattern of reproduction in this species.
- Morphological abnormalities developed in both gradual as well as sudden additions of mercury; but percentage of abnormalities was negligible in gradual addition of mercury as compared to that in sudden addition where 75% (at 100-150 ng/l) – 100% (at 175-275 ng/l) of the specimens were reportedly deformed.
- Though the number of deformed specimens vary considerably in both experiments, the main type of morphological abnormalities remain similar which is significant to characterize the response of *R. leei* to this particular pollutant.

6.6.2. Effect of heavy metal cadmium on *Pararotalia nipponica*

- The specimens subjected to various cadmium concentration attained smaller size than the control specimens.
- Morphological abnormalities developed in all concentration of cadmium; severity of deformation increased with increase in cadmium concentration
- Change in coiling direction was peculiar to the type of morphological deformation of the specimens.

6.7. Significance of the study

Deformities in foraminiferal tests from the polluted environments have been one of the important aspects of pollution monitoring studies utilizing foraminiferal characteristics. Despite the large number of studies monitoring increased deformities in tests from polluted environments, this characteristic is still to attain the status of an effective proxy in pollution monitoring due to the prevailing reports of abnormal tests from naturally stressed environments. There has been the need to differentiate and characterize the benthic foraminiferal response to natural as well as anthropogenic stresses. Laboratory culture studies under controlled conditions where foraminiferal response to single parameters can be monitored effectively is the best solution to address this problem. Present study is an attempt towards this direction where foraminiferal responses to two heavy metals are discussed. The findings confirm the fact that foraminiferal responses can be of significant use to field based studies to decipher marine pollution which is very significant as early warning signals of pollution.

CHAPTER 7

SRESS PROTEINS IN BENTHIC FORAMINIFERA

Chapter 7

Stress proteins in benthic foraminifera

"Success is the ability to go from one failure to another with no loss of enthusiasm."

- Sir Winston Churchill

7.1. Introduction

During unfavorable conditions and stressful situations, many organisms produce increased amount of a small group of specific proteins. These proteins are called stress proteins or heat shock proteins (=Hsps), because the phenomena first observed in drosophila cells when subjected to heat (Tissiere *et al.*, 1974). A number of stressful conditions including environmental (ultraviolet radiation or heavy metals), pathological (infections or malignancies), or physiological (growth factors or cell differentiation) stimuli, induce their expression (Jaattela, 1999). Under such conditions stress proteins bind to partially denatured proteins preventing their aggregation and misfolding, and directly mediate the correct folding (Gething & Sambrook, 1992; Parsell & Lindquist, 1993). Additionally some members of this Hsps-family are expressed constitutively (cognate proteins) (Ashburner, 1982). These forms serve as molecular chaperons in which they support the folding of newly synthesized proteins (Fink, 1999) and facilitate the protein translocation into organelles like the endoplasmatic reticulum, mitochondria, chloroplasts (Haucke & Schatz, 1997) and the lysosome (Chiang *et al.*, 1989).

These processes are very basic physiological mechanisms in living cells, and Hsps must have developed at a very early stage in evolution and they have maintained their structure intact through time. This statement is supported by the fact that Hsps have been found in almost all organisms tested thus far (Pyza *et al.*, 1997), and that the proteins themselves are considered to be highly preserved (Lindquist & Craig, 1988; Gething & Sambrook, 1992). Especially members of the HSP70-family are extremely conservative (Gupta & Golding, 1993; Mukhopadhyay *et al.*, 2003). Comparing the amino acid sequences of human Hsp70 to *Drosophila* Hsp70 and the Hsp70 homologue DnaK of *E. coli* reveals that human Hsp70 is 73% identical to *Drosophila* Hsp70 and 47% identical to *E. coli* DnaK (Hunt & Morimoto, 1985). The comparison of highly conservative Hsp70-protein amino acid sequences or the nucleotide sequence of its gene can be a useful tool in phylogenetic studies (Gupta *et al.*, 1994; Molto *et al.*, 1994;

Germot *et al.*, 1997; Budin & Philippe, 1998; Stedman *et al.*, 1998; Germot & Philippe, 1999; Gribaldo *et al.*, 1999; Gupta *et al.*, 1999; Sulaiman *et al.*, 2000).

Another established application of Hsps based on its inducibility, in which mostly the members of the HSP70- and HSP90- families are used as biomarkers to monitor physiological stress (Köhler *et al.*, 1996; Köhler & Eckwert, 1997). Of them, Hsp70 is known to be the most inducible (Welch, 1993) and most extensively studied stress protein (Mukhopadhyay *et al.*, 2003). The aim of the present study is to

- Verify the presence of Hsp70 in foraminifera which is not done so far.
- To test the expression of Hsp70 in foraminifera as a biomarker for stress.
- Estimate the total protein content of the different benthic foraminiferal species under study which can generally help to estimate the number of individuals needed for immunological investigations

Being a micropaleontologist, it is really difficult to do such sophisticated biotechnological studies on my own. Hence, after setting the aims and objectives of the study, the protein studies were conducted in collaboration with the Animal Physiology group, Tubingen University, Germany. Protein samples were prepared and given and further analyses were conducted by expertise hands of this workgroup. Though I was very much a part of it, doing the immunological analysis on my own was beyond my capacity; hence that part was entirely taken care of by the other people. The analytical methodology is described in the following sections, of which the protocol for immunological studies (though not done by myself) is also incorporated for the convenience of understanding the procedures involved.

7.2. Experimental set up

7.2.1. Foraminifera treatment, protein extraction and concentration

In the laboratory the sediment containing living specimens was transferred in to glass aquaria and maintained under stable conditions of 20 °C temperature, 34‰ salinity, 11:13 h light-dark cycle. Subsamples of sediment were taken from the aquaria and the healthy looking foraminifera full of cytoplasm were picked out with a brush/ pipette, and slightly cleaned with the brush. To determine average size, 15 individuals of each species were photographed with a digital camera attached to a binocular microscope (Z16 Apo, Leica, Germany) and maximum diameter of each individual was measured with image processing software (ImagePro Plus). The isolated and cleaned foraminifera

were transferred into different glass vessels filled with 100 ml of seawater and sealed with parafilm and further subjected to various temperatures (10° C, 20 °C, 35 °C, 45 °C) in order to test the activation of proteins under different stress levels.

For the extraction of the proteins, the living foraminifera were taken out of the vessels, cleaned in sea water with a brush, and transferred with the smallest possible amount of water to an eppendorf tube. Extraction buffer (80 mM potassium acetate, 5 mM magnesium acetate, 20 mM Hepes pH 7.5) and proteaseinhibitor (Protease Inhibitor Cocktail, Produkt Nr. P8340, Fa. Sigma) were added and the foraminifera were homogenized in the eppendorf tube with a plastic pestle (Fig.7.1). To get rid of the shells, the homogenate was centrifuged at 4 °C at 1200 rpm for 10 minutes and the supernatant was transferred to a new eppendorf tube.

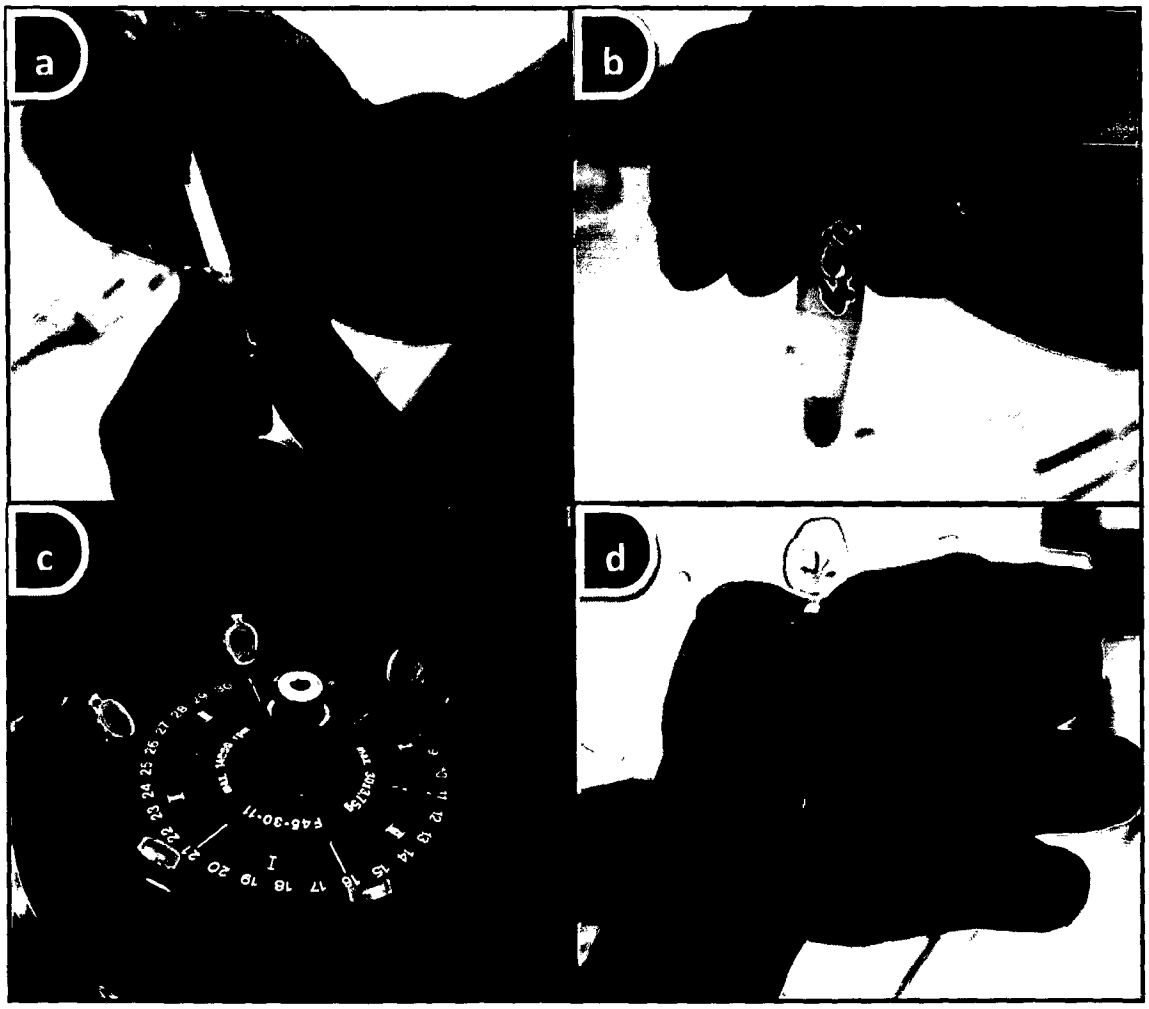


Fig. 7.1: Steps involved in the extraction of proteins:
a) homogenising the foraminifera with a plastic pestle; **b)** the test fragments settled at the bottom of the tube; **c)** centrifuging the mixture; **d)** clear supernatent transferred to a new tube.

For the analysis of the protein content, 4 µl of the protein solution was kept for the Bradford test. The rest of the protein solution was mixed with sodium dodecyl sulfate (SDS) -buffer (20% glycerine, 3% SDS, 0.3% s-mercaptoethanol, 10 mM tris ph7) 2:1 ratio, cooked at 95 to 100 °C for five minutes, and kept for further steps at -20°C.
Ultrafiltration units for centrifuges (Roti®-Spin Mini-30, Fa. Roth) with a nominal molecular weight cut off of 30 kDa were used for concentrating the proteins after the extraction (Fig. 7.2). This procedure detained all proteins bigger than 30 kDa over the filters, which lead to an accumulation of Hsp70 in the supernatant.

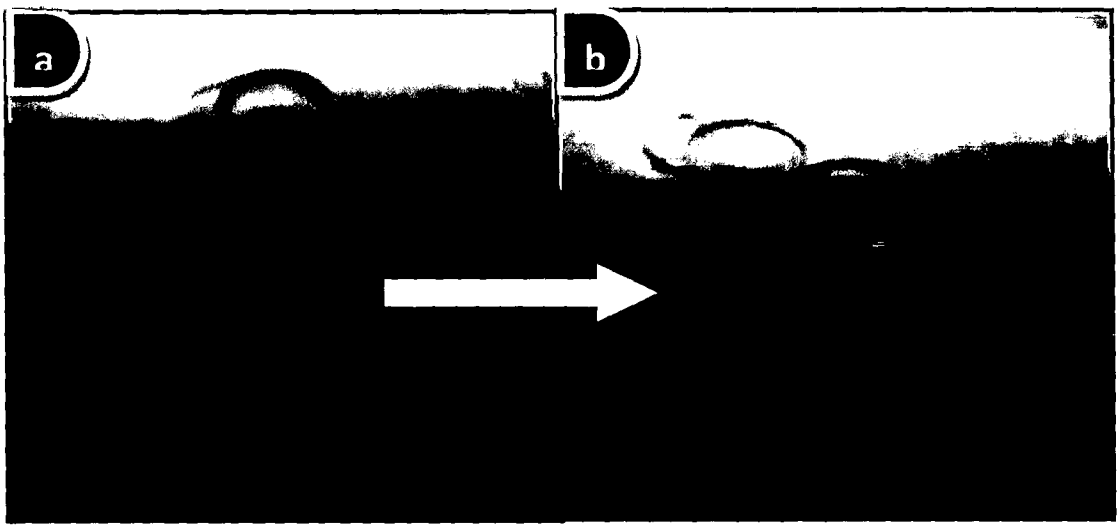


Fig. 7.2: Ultra filtration units for centrifuges used for concentrating the proteins:
a) Sample reservoir; **b)** membrane: **c)** filtrate reservoir

7.2.2. Bradford test

The protein content of the foraminifera samples was obtained from two measurements per sample. For each measurement 2 µl of the protein solution together with 23 µl of extraction buffer 1:10 (80 mM potassium acetate, 5 mM magnesium acetate, 20 mM Hepes pH 7.5) were transferred to one well of a 96-well microplate. For the standard curve a serial dilution of Bovine serum albumin (BSA) (0.4 mg/ml) in extraction buffer 1:10 was used. After the wells were filled with the sample probes and the standards, 250 µl of Bradford-Reagent was added in each well. Measurements were made with an automated microplate reader (Automated Microplate Reader, Elx 8006, Bio-Tec Instruments, Germany).

7.2.3. Immunological analysis *(Performed by the animal physiology group, Tubingen University, Germany)*

Immunological analysis was done following the protocol of Köhler *et al.*(2005). Extracted proteins were analyzed by minigel SDS-PAGE (12% acrylamide (w=v), 15 min at 80 V and 90 min at 120 V). Using the method of Western blotting (Burnette, 1981), protein was transferred to nitrocellulose by semi-dry blotting, and the filter was blocked for 2 h in 50% horse serum in TBS (50 mM Tris pH 7.5, 150 mM NaCl). After washing in TBS, monoclonal antibody ("Heat Shock Protein70 (MA3-006)"; Dianova, FRG, dilution 1:5000, respectively "Anti-Hsp70/Hsc70 (SPA-822)"; Dianova, FRG, dilution 1:1000; Dianova, or Anti-Hsp70/Hsc70 (SPA-820)"; Dianova, FRG, dilution 1:1000 in 10% horse serum=TBS) was added and the sample was then incubated at room temperature overnight.

After repeated washing in TBS for 5 min, the nitrocellulose filter was incubated in the secondary antibody solution (goat anti-mouse IgG coupled to peroxidase, Dianova, FRG, dilution 1:1000 in 10% horse serum=TBS) at room temperature for 2 h. After subsequent TBS washing, the antibody complex was detected by the staining solution (1 mM 4-chloro (1) naphthol and 0.015% H_2O_2 in 30 mM Tris pH 8.5 containing 6% methanol).

To quantify the amount of protein in the bands the optical volumes (area (number of pixels) x average grey scale value) of the Western blot protein bands were measured after background subtraction with a densitometric image analysis system (Herolab E.A.S.Y.). For comparison, the optical volumes were normalized by dividing them through the amount of total protein transferred to each lane.

7.3. Results

7.3.1. Protein contents

Protein concentrations of 4 benthic foraminiferal species were analyzed by the Bradford test. Table 7.1 summarizes the measured amount of proteins for different species and also the protein per individual calculated by dividing the total measured by the total number of individual specimens used for the experiment. In case of species where more than one trial was conducted, average amount of total protein was calculated.

The average size of all the four species analyzed for protein contents was measured in order to observe the relationship between test size and biomass volume (Table 7.2).

Foraminiferal species	Total protein (µg) (A)	Number of individuals used (B)	Total Protein per Individual (µg) (C=A/B)	Total Protein per Individual (µg) Average
Ammonia tepida	80.300	1300	0.06177	**0.0661**
Ammonia tepida	109.074	1300	0.08390	
Ammonia tepida	82.365	1300	0.06336	
Ammonia tepida	175.820	3170	0.05546	
Massilina secans	40.954	175	0.23402	
Massilina secans	18.451	70	0.26359	**0.2314**
Massilina secans	13.755	70	0.19651	
Ammonia beccarii	44.171	293	0.15075	**0.1508**
Elphidium crispum	24.285	600	0.04048	**0.0405**

Table 7.1: Total protein content measured and total protein content per individual for different species

Foraminiferal species	Average size (µm)	Total Protein per Individual (µg)
Massilina seccans	1154.08	0.2314
Ammonia beccari	645.09	0.1508
Ammonia tepida	445.83	0.0661
Elphidium crispum	543.07	0.0405

Table 7.2: Average size and average total protein content per individual of the different species

7.3.2. Hsp70-Immuno Assay

Protein extractions of different species, stress temperatures and incubation times were tested in several trials to analyze the expression of Hsp70. Only during one trial, a weak band of Hsp70 was obtained, where 357 specimens of *A. Beccarii,* 300 specimens of *E. Crispum* and 386 specimens of *M. secans* were pooled together for the analysis.

Later on, the methodology of the experiment was modified in comparison to the previous attempts -

- Instead of mixing different species for analysis, single species *Ammonia tepida* was used in more number so as to make sure that the expression is clearly of this particular species.
- Modified the stress temperatures: 1300 specimens each were kept in replicate at 10° C, 20 °C, 35 °C, 45 °C temperature for 4 hours.

- Proteins were concentrated after extraction (before transferring the protein solution into the gel) with a protein filter. This allowed significantly more protein into the pockets of the gel. This modification enhanced the detection of Hsp70 by producing visible bands significantly (Fig. 7.3).
- Two primary antibodies were still used ("Heat Shock Protein70 (MA3-006)"; "Anti-Hsp70/Hsc70 (SPA-822)").

7.3.3. Temperature Dependant Expression

As explained above, four sets of specimens with replicate (1300 *Ammonia tepida* specimen in each set) were prepared and exposed to different temperatures: 10° C, 20 °C, 35 °C, 45 °C in the experiment. The amount of total protein filled into each lane and other experimental details are tabulated in Table 7.3. The corresponding section of the filter is given as Figure 7.3. No intact proteins were detected by the Bradford test in the protein solution treated with 45°C (refer lane 5 devoid of any bands of Hsp).

Filter lane	Sample used	Temperatures	Amount of total protein analysed for Hsp70 (µg)
1	*D. rerio* (standard)		40.0
2	1300 *A. tepida*	4h / 10°C	77.9
3	1300 *A. tepida*	4h / 20°C	106.0
4	1300 *A. tepida*	4h / 35°C	79.8
5	1300 *A. tepida*	4h / 45°C	---

Table 7.3: Details of the sample, temperature provided to induce Hsp, and the total protein content analyzed for Hsp 70

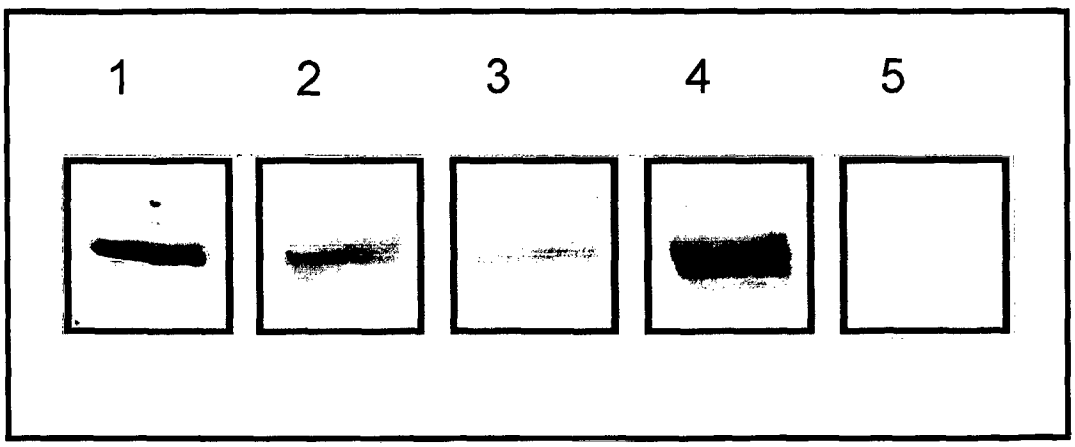


Fig. 7.3: Filters showing bands which Indicate the presence of Hsp 70: lane **1**: Standard (*D. rerio*); lane **2**: *A. tepida* @ 10°C; lane **3**: *A. tepida*, @ 20°C; lane **4**: *A. tepida* @ 35°C; lane **5**: *A. tepida* @ 45°C

To make the bands of lane 2 - 5 comparable, their optical volumes were normalized by dividing each value through the corresponding transferred protein mass. The amount of detected Hsp70 was lowest at 20 °C (lane 3: which was also the normal unstressed culture temperature), followed by 10 °C. The highest expression of Hsp 70 was found at 35 °C (lane 4). The expression of *A. tepida* against each temperature is plotted in Figure 7.4.

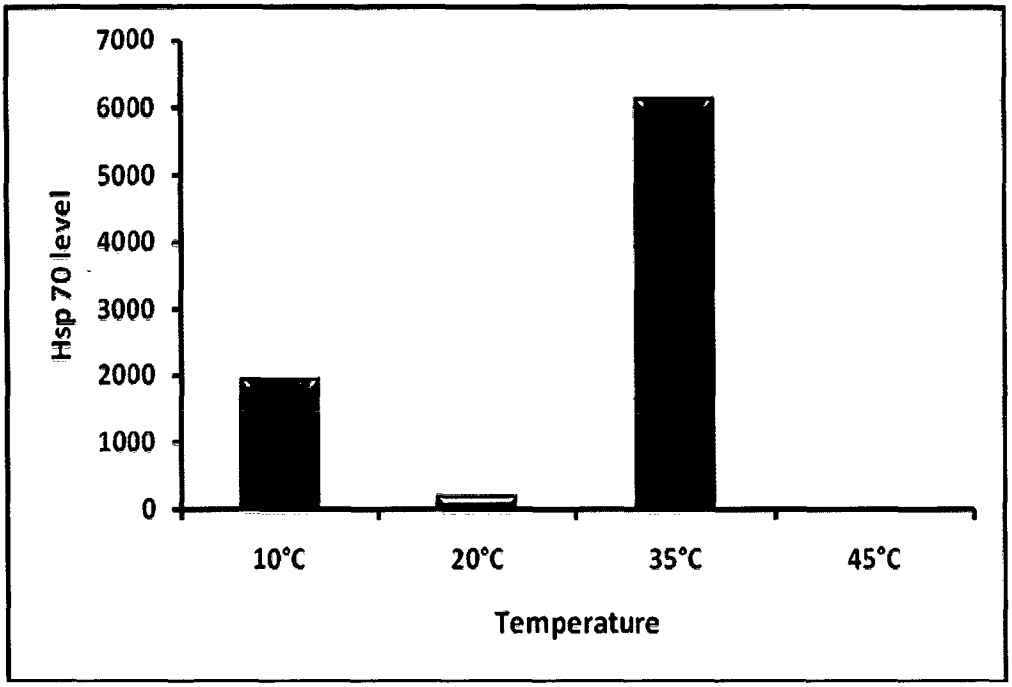


Fig. 7.4: Temperature dependant expression pattern of *Ammonia tepida*

7.4. Discussion

7.4.1. Protein contents

Average protein concentration varies from 0.231 µg/cell and 0.040 µg/cell between species. Attempts to find values of protein concentrations of other protists in the literature to compare with the present study failed. Most probably, this is the first time that the protein content in foraminiferal cells was analyzed. The results are helpful in pre-estimating the number of specimens needed for immunological analysis on benthic foraminifera. Such evaluations are important in the case of foraminifera, because in contrast to many other protists it is difficult to grow a sufficient amount of foraminifera in culture and the number of living specimen in samples widely varies. Furthermore, the method of separating the foraminifera from the sediment is time-consuming because single specimens must be picked out of the sediment.

When the mean protein content per individual is compared with the average size of the corresponding species, most values correlate very well except for *Elphidium crispum*. The relatively low total protein content of this species can be explained by its relatively thick shell plus an extended canal system within its shell, which highly reduces the space available for cytoplasm. The protein content not only depends on size, but also on the thickness and shape of the shell and the inner structure of the chambers. Another factor that may strongly influence the total protein content is the physiological state of the foraminifera during the experiment.

7.4.2. Hsp70-Immuno assay

So far, the only organisms in which Hsp70 (DnaK) was not found were some members of the phylogentically remote group of Achaeans (Gribaldo *et al.* 1999). Some marine organisms in the Antarctic had lost their Hsp70 stress response, probably due to the constant low temperature of the surrounding water (Brennecke *et al.*, 1998; Hofmann *et al.*, 2000). During our search for Hsp70 in benthic foraminifera, continuously small methodological changes were made between the different experiments. The modification with the greatest success was the introduction of an additional concentration step of the protein solution (see methods).

About 50 µg of the total protein is needed to detect Hsp70 in foraminifera, which is remarkably more than that used in the majority of studies dealing with other organisms (Knigge & Köhler, 2000; Köhler *et al.*, 2000; Bayley *et al.*, 2001). This can be explained by a considerably increased level of other essential proteins in foraminiferal cells, like actin and tubulin, which then lead to a proportionally low concentration of Hsp70 in the cytoplasm. Actin and tubulin are structural proteins and basic components of the cytoskeleton of each eukaryotic cell. Pseudopodial movement and the high dynamic cytoplasm motility of foraminifera need the presence of many microfilaments and microtubules composed of actin and tubulin (Travis & Bowser, 1986; Bowser *et al.*, 1988).

Another explanation for the high amount of protein required to detect Hsp70 in benthic foraminifera might be the reduced ability of the used primary antibodies to bind to foraminiferal Hsp70. Three different primary antibodies were used during the search for foraminiferal Hsp70. The antibodies were developed to bind a large variety of Hsp70 proteins from different species, as the target epitope of the amino acid sequence for the

antibody to bind is located in the highly conservative ATP-binding region of the protein. Yet as foraminifera are phylogenetically quite far away from most of the other organisms tested with these antibodies, binding may be impeded by small variations in the sequence. The following organisms tested positive with the corresponding antibody: "Heat Shock Protein70 (MA3-006)"binds with yeast, Drosophila, fish, mouse, avian, amphibian human samples and *Chlamydomonas reinhardtii* (Bloch & Johnson, 1995; Yang *et al.*, 2008).

"Anti-Hsp70/Hsc70 (SPA-822)" binds to human, mouse, rat, beluga, bovine, canine, chicken, Drosophila, fish, guinea pig, hamster, monkey, mussel, pig, rabbit, scallop, sheep, Xenopus, yeast and *Euglena gracilis* (Barque *et al.*, 2000). "Anti-Hsp70/Hsc70 (SPA-820)" binds to human, mouse, rat, beluga, bovine, canine, chicken, fish (carp, chinook salmon, chum salmon, rainbow trout), guinea pig, hamster, monkey, mussel, pig, plant (cucumber, pea), rabbit, sheep and Xenopus (compare product data sheet).

If the foraminiferal epitope is quite different from the equivalent of the epitope of the immonogen, the antibody was designed with (for "Heat Shock Protein70 (MA3-006)" and "Anti-Hsp70/Hsc70 (SPA-822)": human Hsp70, for "Anti-Hsp70/Hsc70 (SPA-820)": chicken Hsp70), it is possible that the binding is very weak or even does not take place at all.

7.4.3. Temperature dependent expression

Experiment was conducted to investigate the relationship between stress temperature and expression of the Hsp70 protein. Individuals from one single species (*A. tepida*) were analyzed in this experiment. Four identical sets of foraminifera were prepared and exposed to four different temperatures. It was not possible to extract the same amount of total protein from each set. Because it was decided to transfer the maximum amount of total protein available from each set to enhance the likeliness to have enough Hsp70 for detection, the amount of total protein transferred to the different lanes was not equal. To get comparable data from the resulting filter, the resulting optical volumes of the bands on the filter were normalized by dividing them through the amount of total protein transferred to each lane.

As shown in Figure 7.3, the expression of Hsp70 was highest at 35 °C. At the culture temperature of 20 °C almost no Hsp70 was found, but at 10 °C the formation of Hsp70 again increased.

These results fit very well to the established statement that Hsp70 is induced by denaturated proteins caused by physiological stress (Voellmy & Goldberg, 1981; Palleros *et al.*, 1991), which can be created by higher temperatures as well as lower temperatures (Creighton, 1990). The results also match to the Hsp70 reaction curve of Eckwert *et al.*,(1997), showing the relationship of stress intensity and Hsp70 level. No Hsp70 was detected at 45°C and also the Bradford analysis did not detect any intact proteins.

No data was found about the maximum tolerable temperature of *A. tepida,* but as the optimal reproduction temperature of *A. tepida* of 23.5 – 24 °C (Rao & Rao, 1974) and their culture temperature of 20 °C were exceeded by more than 20 °C; it is assumed that the consequences for the foraminifera must have been severe. An increase of the temperature this high above the optimal temperature suggests that the foraminifera did not survive the heat and the proteins have possibly been destroyed.

7.5. Conclusions

From the present study the following conclusions were drawn-

- The amount of total protein per individual of the four benthic foraminiferal species studied as a part of this study showed the following trend- *M. secans* (0.231 µg), *A. beccarii* (0.085 µg), *A. tepida* (0.066 µg) and *E. crispum* (0.040 µg).
- The mean protein contents per individual are positively correlated their average size in all 3 species other than *E. crispum.*
- The stress protein Hsp 70 was positively tested for benthic foraminiferal species *A. tepida*
- The relationship between stress temperature and expression of the Hsp70 protein shows that the Hsp70 is induced by denaturated proteins caused by physiological stress (higher temperatures as well as lower temperatures).

7.6. Significance of the study

Results indicate that foraminifera contain Hsp70 and that it has a temperature dependent expression pattern caused by changing stress conditions. This offers several different new applications. Foraminiferal Hsp70 expression can now be used as a biomarker for stress and it is possible to investigate tolerance ranges of different species towards different environmental factors or pollution. Using foraminiferal Hsp70 as a bioindicator for stress also has its limitations because a high amount of biomass is

needed for the procedure and large numbers of specimen of the corresponding species have to be available.

Future work on foraminiferal Hsp 70 should not be restricted to stress depending expression. Sequences of Hsp70 encoding genes could be used for phylogeny. Until now molecular phylogenetic studies within the group of foraminifera have been commonly based on the small subunit rRNA (SSU) genes (Darling & Wade, 2008; Schweizer *et al.*, 2008).

These genes can easily be obtained from single cells isolated from environmental samples (Flakowski *et al.*, 2005). Drawbacks of the SSU genes are their heterogeneity of substitution rates biasing the phylogenies (Philippe, 2000) and their low resolution at high level relationships (Flakowski *et al.*, 2005). Due to their extreme conservativeness, Hsp70 encoding genes are expected to be more suitable for high level relationships than the SSU rRNA. In any case sequences of Hsp70 encoding genes could provide an important alternative source of information. The extraordinary fossil record of foraminifera would provide the best conditions for testing this method. There are numerous studies in which Hsp70 genes are used for phylogenetics (Gupta *et al.*, 1994; Molto *et al.*, 1994; Germot *et al.*, 1997; Budin & Philippe, 1998; Stedman *et al.*, 1998; Germot & Philippe, 1999; Gribaldo *et al.*, 1999; Gupta *et al.*, 1999; Sulaiman *et al.*, 2000) but none of them within the group of foraminifera.

CHAPTER 8

FORAMINIFERAL RESPONSE TO ^{13}C LABELED FOOD

Chapter 8

Foraminiferal response to ^{13}C labeled algal food

"Once 'what' is decided, 'how' always follows. We must not make 'how' an excuse for not facing and accepting the 'what' "

– Pearl S. Buck

8.1. Introduction

Benthic foraminifera form a large part of the biomass of the benthic community (Heip *et al.*, 2001) of marine realm and are important proxies for paleoenvironmental studies owing to their extensive fossilization potential. At times they comprise more than 50% of the benthic biomass (Snider *et al.*, 1984; Gooday *et al.*, 1992) and therefore play a significant role in the carbon budget of the oceans. Oceans hold about 60 times more carbon than the atmosphere; a small change in the chemistry of oceans can produce a marked change in atmospheric reservoir. There are number of ways by which the ability of the ocean to hold carbon dioxide can change. Out of several factors, ocean productivity helps to control the partitioning of carbon between large ocean reservoir and the relatively small atmospheric reservoir (Berger *et al.*, 1989). Therefore, productivity changes play an important role in providing feedback to climatic changes.

Primary production is carried out by marine plants mainly microscopic algae (phytoplankton) and microphytobenthos (diatoms, cyanobacteria, etc). They produce organic matter by converting inorganic carbon to organic carbon through photosynthesis using various nutrients and sunlight as energy source. Marginal marine environments receive nutrients both from freshwater inflow and from the decomposition of organic matter in the sediments. The movement of water through wave and tidal circulation keeps mixing the nutrients in the water column and promotes enhanced primary production (Murray, 2006).

There are many previous reports which strongly correlate the benthic foraminiferal assemblages with the surface ocean productivity and subsequent changes in the organic carbon flux (Corliss & Emerson, 1990; Gooday & Turley, 1990; Ohga & Kitazato, 1997; Gooday & Rathburn, 1999; Altenbach *et al.*, 1999; Kitazato *et al.*, 2000; Fontanier *et al.*, 2002, 2003).

Many strategies by which benthic foraminifera can feed upon the organic matter have been proposed (reviews by Lee, 1980; Lipps, 1983; Goldstein, 1999). However quantitative estimation of the rate of ingestion of organic carbon by benthic foraminifera has remained scarce. Until now the determination of feeding behavior of benthic foraminifera was mainly circumstantial; if the organism gathers a particular food, it was normally assumed to feed on it. The TEM studies of protoplasm, feeding experiments and biomarker analysis can help confirm the feeding habits of benthic foraminifers (Murray, 2006).

Feeding experiments conducted in the laboratory are a conventional way to examine how benthic foraminifera react to small amounts of organic matter on a time scale of a few days (Heinz *et al.* 2001, 2002). The stable isotopes are useful tracers for following the fate of labeled material over short to long time scales and for quantitatively examining the ingestion rates of benthic organisms (Blair *et al.,* 1996; Levin *et al.*, 1999, Middelberg *et al*, 2000).

In the present study attempt is made to observe the differential uptake of ^{13}C labeled food (*Dunaliella tertiolecta*) by benthic foraminiferal species *Ammonia tepida* with time. For this purpose, experiment was conducted using stable isotope ^{13}C labeled *Dunaliella tertiolecta* which was added to the experimental dishes at the onset of the experiment and observations were made after 2 days, 7 days, 21 days and 42 days.

8.2. Experimental set-up

Benthic foraminiferal species *Ammonia tepida* (Fig. 8.1) collected from the Bay of Aiguillon (as per the methods described in chapter 3) was studied for its response to ^{13}C labeled algal food. This species was the most abundant and was selected because large number of live specimens was required for the present study.

Taxonomic status:

Order: *Foraminiferida* (Eichwald, 1830)

Suborder: *Rotaliina* (Delage & Herouard 1896)

Family: *Rotaliidae* (Ehrenberg, 1839)

Genus: *Ammonia* (Brunnich, 1772)

Species: *Ammonia tepida* (Cushman 1926)

Fig.8.1: Live specimen of *Ammonia tepida*

Freeze dried, labeled *Dunaliella tertiolecta* provided by the Micropaleontology laboratory, Geosciences department, Tubingen University was used as food. *Dunaliella tertiolecta* is a unicellular algae belonging to *Chlorophyceae*. *Dunaliella* has been used as the representative of phytoplankton by previous workers. The fast and active response of foraminifera to *D. tertiolecta* has been reported from the laboratory as well as field studies in Sagami bay by Kitazato *et al.* (2003), Nomaki *et al* (20005a, 2005b), and in Mediterranean Sea by Heinz *et al* (2002).

In order to assess the differential uptake of labeled algal food with time, 4 sets of specimens in replicates were prepared which were incubated for different time periods viz. 2 days, 7 days, 21 days, 42 days (Table 8.1). Each experimental dish contained 500 specimens in 100 ml of seawater in to which the labeled algal food was introduced at the onset of the experiment. Salinity (34±1‰) and dissolved oxygen (7±1 ml/l) were kept constant throughout the experiment. All the experimental trays were incubated at 20°C throughout the experiment with a 12hour light 12hour dark illumination cycle.

Once the incubations were over, the dishes were taken out of the incubator and samples were prepared for various analyses. The foraminifera were carefully removed from the experimental dishes to ependorf cups with minimum possible water. A total of 350 out of the total 500 specimens in each set were kept for the biomass analysis. The remaining 150 specimens were used for the ^{13}C analysis of foraminiferal tests.

Experiment sets	No. of specimens	Date of start	End date	Days of Incubation
Ammonia-42 set 1	500	28.8.08	9.10.08	42 days
Ammonia-42 set 2	500	28.8.08	9.10.08	42 days
Ammonia-21 set 1	500	03.09.08	24.09.08	21 days
Ammonia-21 set 2	500	03.09.08	24.09.08	21 days
Ammonia-7 set 1	500	12.09.08	19.09.08	7 days
Ammonia-7 set/2	500	12.09.08	19.09.08	7 days
Ammonia-2 set 1	500	17.09.08	19.09.08	2 days
Ammonia-2 set 2	500	17.09.08	19.09.08	2 days

Table 8.1: Details of the experimental sets of the study to decipher the response of *Ammonia tepida* to ^{13}C labeled food (Set 2 corresponds to the replicate of each set)

The analysis was done at Museum für Naturkunde Leibniz-Institut für Evolutions- und Biodiversitätsforschung an der Humboldt-Universität zu Berlin. The samples were pretreated for both biomass and ^{13}C analysis. As per the following procedure-

- Biomass analysis: For the biomass analysis, the foraminiferal specimens were initially cleaned 2-3 times in artificial seawater and then the samples were treated with 10% hydrochloric acid to get rid of the calcareous test, till the bubbling stops from the epindorf cups. Afterwards, the samples were heated in an oven for 2 days at 50°C. Once dry, the samples were transferred to silver cups to be sent for analysis.
- Hard part analysis: For the analysis of foraminiferal test, the experiment specimens were initially cleaned 2-3 times in artificial seawater and then the samples were treated with 10 % hydrogen peroxide and further ultrasounicated for a few seconds to remove the organic material. Once the tests were cleaned, they were transferred in to pre-labeled round punch slides and send for analysis.
- The excess ^{13}C was calculated for each sample using the following formula and plotted against time

$$Excess = \delta\,^{13}C \text{ labeled sample} - \delta^{13}C \text{ natural sample (background)}$$

8.3. Results and discussion

The excess was calculated and plotted against time for the hard parts as well as for the biomass of *Ammonia tepida* (Fig. 8.2 & Fig. 8.3).

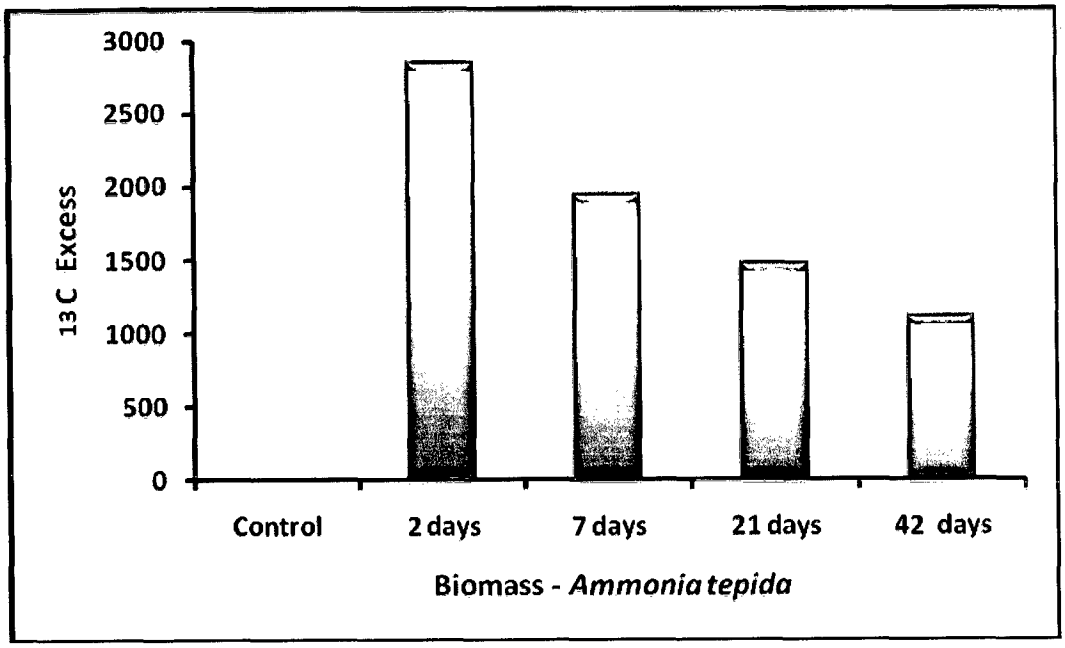


Fig.8.2: Graph showing the relation Excess ^{13} C (in biomass) of *Ammonia tepida*

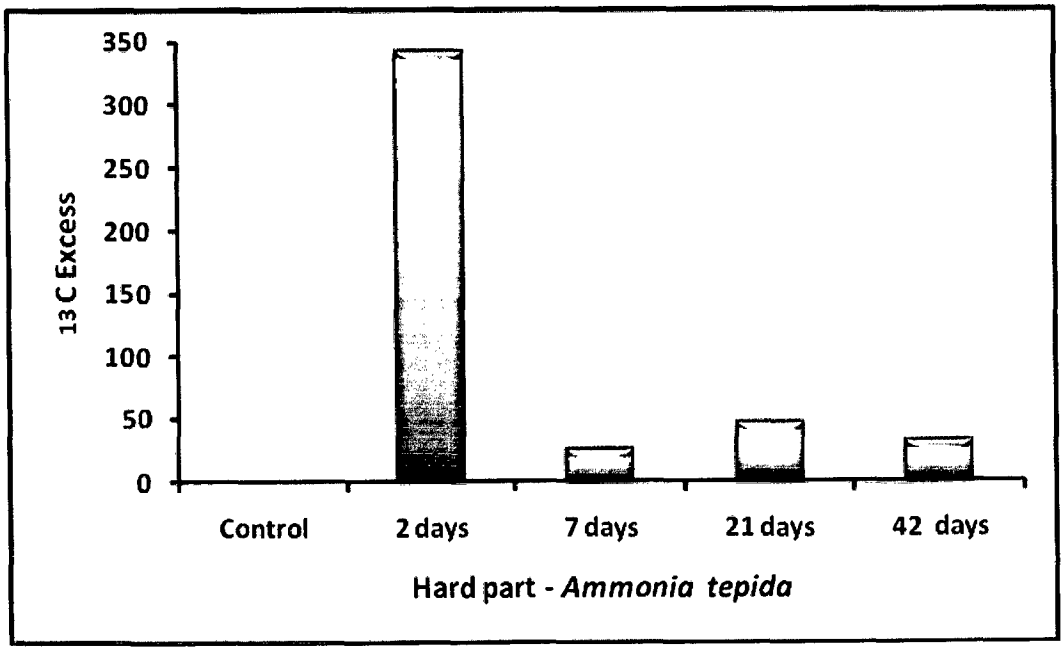


Fig.8.3: Graph showing the relation Excess ^{13} C (in hard part) of *Ammonia tepida*

The ^{13}C excess was found to follow a similar trend in both the biomass and hard part of *Ammonia tepida*. Excess was found maximum in the biomass as well as the hard part of *Ammonia tepida* on the sample incubated for two days. And thereafter the ^{13}C excess reduced with time; the trend is steady in the biomass of *Ammonia tepida* whereas in the hard part, only the value on day 21 showed a slight variation. The decreasing ^{13}C excess with time indicates that the foraminifera ingested food quickly, during the initial days and thereafter the ingestion rates steadily decreased with time from day 7 to day 21 and then finally the lowest values on day 42.

The faster uptake of labeled food by the foraminifers shows the affinity of this species to fresh organic matter/food material. With time, it is expected that the labeled organic carbon starts to degrade which might have caused the lowering of ^{13}C signals with elapsed time. Some previous ^{13}C labeling experiments using algal material found that certain parts of the added algae were degraded after a few hours to days (Middelburg *et al.*, 2000; Thomas & Blair, 2002; Moodley *et al.*, 2005). *Ammonia tepida* is reportedly a herbivore, not a detritivore (Murray, 1991; Burone *et al.*, 2007), hence the affinity to freshly added organic matter/food can possibly be justified.

The part of the decrease in the ^{13}C signals of the biomass can also be attributed to the utilization of the ingested material for various metabolic activities and especially for secreting the hard part which is commonly known as test. Kuile *et al.* (1987) observed that less than 5 % of the carbon taken up by the foraminifera through feeding is incorporated in to the skeleton; rest is retained in the organic fraction. It is evident from the graph that comparatively very less amount of carbon has been incorporated in to the tests than that of the biomass (Fig. 8.2 & 8.3). Lastly part of the decrease in the ^{13}C excess with time can also be attributed to the excretion and loss through respiration with time. The high initial uptake suggests that the algal food taken up by the pseudopodial network is immediately transported inwards. The uptake continues till the foraminifera are filled to the capacity with food, resulting in a strong decline of feeding rates even if food is still available. A considerable amount of the food may be ejected after partial digestion and the rate of execretion vary in different species. It is also possible that the foraminifera excrete more than 50 % of the carbon derived from the food within 24 hour as previously observed in the case of *A. lobifera* (Kuile *et al.*, 1987). The results of the present study on *Ammonia tepida* are in line with the views expressed by Kuile *et al.* (1987) and the earlier microscopic observations by Lee (1974) and Koestler *et al.* (1984).

8.4. Conclusions

Based on the experiment on 4000 live specimens of *Ammonia tepida*, the following conclusions were made-

- *Ammonia tepida* prefers fresh organic matter to decayed organic matter as its food material.
- There is a quick initial uptake of food material by *Ammonia tepida* and thereafter the uptake decreases with time
- *Ammonia tepida* utilized a small fraction of the incorporated food for secreting the test; the rest was retained in the biomass
- Considerable amount of excretion of the digested food and also loss of carbon through respiration may be pliable reason for the steady decline in the ^{13}C signals with time

8.5. Significance of the study

It is now widely accepted that organic carbon flux is one of the main factors that control the distribution patterns of benthic foraminifera. In contrast to numerous investigations of faunal and abundance changes among benthic foraminifera, feeding ecologies in relation to phytodetritus processing is not well understood. Experimental studies in which food material is introduced can enable observations on the feeding behavior of foraminifera on different time scales which in turn are very useful in various paleoceanographic interpretations.

Chapter 9

Conclusions and future prospective

"It is never safe to look into the future with eyes of fear"

-Edward Henry Harriman

9.1. Conclusions

The following conclusions were drawn from the various experimental studies on benthic foraminifera compiled in this thesis. For ease in description and understanding, the conclusions are enlisted under separate subheadings.

9.1.1. Growth and reproduction in benthic foraminifera:

9.1.1.1. *Rosalina leei*

- Under the given laboratory conditions, *Rosalina leei* underwent only asexual reproduction in all the three consecutive generations.
- The number of juveniles formed from a single mother cell varies from 40-60 in number and the juveniles were born with 3-4 chambers.
- Prior to reproduction *Rosalina leei* made a reproductive cyst surrounding the test with the pseudopodial network.
- Lifespan of *Rosalina leei* was observed to be 105-109 days.

9.1.1.2. *Strebloides advena*

- Under the given laboratory conditions, *Strebloides advena* underwent only asexual reproduction in all the 7 consecutive generations.
- The number of juveniles formed from a single mother cell varied from 15-20 in number and the juveniles are born with 3-4 chambers
- In *Strebloides advena* the process of making reproductive cyst was not noticed prior to reproduction
- Lifespan of *Stebloides advena* was observed to be 17-19 days.

9.1.2. Foraminiferal response to different oxygen levels

- Any change in natural oxygen conditions affects the foraminiferal abundance.

- Infaunal assemblages are more adaptive to changed oxygen conditions in contrast to epifaunal assemblages, which quickly die out.

9.1.3. Foraminiferal response to heavy metal pollutants

9.1.3.1. Effect of heavy metal mercury on *Rosalina leei*

- The response of benthic foraminifera *Rosalina leei* is different and distinct to gradual and sudden stress conditions.
- On gradual increase in the mercury concentrations, specimens showed lesser growth with increasing Hg concentration.
- Sudden addition of mercury increased stress induced reproduction which is significantly different from the normal pattern of reproduction in this species.
- Morphological abnormalities developed in both gradual as well as sudden additions of mercury; but percentage of abnormalities was negligible in gradual addition of mercury as compared to that in sudden addition where 75% (at 100-150 ng/l) – 100% (at 175-275 ng/l) of the specimens were reportedly deformed.
- Though the number of deformed specimens vary considerably in both experiments, the main type of morphological abnormalities remain similar which is significant to characterize the response of *R. leei* to this particular pollutant.

9.1.3.2. Effect of heavy metal cadmium on *Pararotalia nipponica*

- The specimens subjected to various cadmium concentration attained smaller size than the control specimens.
- Morphological abnormalities developed in all concentration of cadmium; severity of deformation increased with increase in cadmium concentration
- Change in coiling direction was peculiar to the type of morphological deformation of the specimens.

9.1.4. Stress proteins in benthic foraminifera

- The amount of total protein per individual of the four benthic foraminiferal species studied as a part of this study showed the following trend- *M. secans* (0.231 µg), *A. beccarii* (0.085 µg), *A. tepida* (0.066 µg) and *E. crispum* (0.040 µg).

- The mean protein contents per individual are positively correlated their average size in all 3 species other than *E. crispum.*
- The stress protein Hsp 70 was positively tested for benthic foraminiferal species *A. tepida*
- The relationship between stress temperature and expression of the Hsp70 protein shows that the Hsp70 is induced by denaturated proteins caused by physiological stress (higher temperatures as well as lower temperatures).

9.1.5. Foraminiferal response to ^{13}C labeled food

- *Ammonia tepida* prefers fresh organic matter to decayed organic matter as its food material.
- There is a faster initial uptake of food material by *A.tepida* thereafter the uptake decrease with time
- *A. tepida* utilized a small fraction of the incorporated food for test building; the rest was retained in the biomass
- It is assumed that there was a considerable amount of ejection of the digested food and also loss of carbon through respiration which caused a steady decline in the ^{13}C signals with time

9.2. Future prospective

During the span of this research work considerable amount of time was spent on establishing the techniques, facilities etc, and the present thesis is just the beginning of a journey I passionately undertake as my career. Though I am happy of what could be done so far, much more lies ahead; the following are some of the stepping stones-

- Elemental analyses of the foraminiferal tests: The experimental results would be more refined if the elemental studies on the foraminiferal tests are carried out as it would give the clear understanding of the change in the chemical composition as a result of the parameter under consideration. This hold more effective for the studies related to heavy metals in order to quantify the amount of intake of heavy metals by the calcareous test of foraminifera, but equally holds well in case of experiments on natural parameters like temperature, salinity, oxygen manipulations.

- Tracer experiments: Tracers will help tracking the pathways and preferences in the uptake of the parameter under observation. It also gives a quantitative record along with the qualitative observations.

- Methane & organic matter experiments: Foraminiferal response to methane seeps are being attempted these days in insitu experiments by some of the foreign workers. Attempting refined experiments in the field conditions in Indian waters and laboratory conditions simultaneously will help establishing this particular application of benthic foraminifera.

- Crystallographic studies: Similar to the elemental changes in the foraminiferal test as a result of various parameters, it is expected that the crystallographic arrangement of the test is also affected. It would be very useful if such studies are carried out on experimental studies n order to establish this new foraminiferal proxy for various studies.

With a positive note, I wind up this thesis:

"Far away in the sunshine are my highest inspirations. I may not reach them, but I can look up and see the beauty, believe in them and try to follow where they lead."

– Louisa May Alcott

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ANNEXURES

Annexure 1

Manuscripts

1. Panchang, R., Nigam, R., **Linshy, V.N.**, Rana, S.S. & Ingole, B. (2006) Effect of oxygen manipulations on benthic foraminifera: A preliminary experiment, Indian Journal of Marine Sciences, 35(3), 235-239.
2. **Linshy V.N.**, Kurtarkar, S., Rana, S.S., Saraswat, R. & Nigam, R. (2007) Appraisal of laboratory culture experiments on benthic foraminifera to asses/ develop paleoceanographic proxies, Indian Journal of Marine Sciences, 36(4), 301-321.
3. R. Nigam, Sujata R. Kurtarkar, R. Saraswat, **Linshy V. N.** & S. S. Rana (2008) Response of benthic foraminifera Rosalina leei to different temperature and salinity, under laboratory culture experiment, Journal of Marine Biological Association U.K., 88(4), 699-704.
4. R. Nigam., **Linshy V. N.**, Sujata Kurtarkar & R. Saraswat (2009). Effect of sudden stress due to heavy metal mercury on benthic foraminifera Rosalina leei: Laboratory culture experiments, Marine Pollution Bulletin, 59, 362-368.
5. Marten, R., **Linshy V.N.**, Haap, P., Geslin, E., Kohler, H.R., Heinz, P., Total protein contents and analysis for the stress protein Hsp 70 in living shallow water benthic foraminifera *(Communicated)*
6. **Linshy, V.N.**, Sujata R.K., & Nigam R. Growth and reproduction in benthic foraminiferal species Strebloides advena: observations from laboratory culture studies (Under Review with Journal of Foraminiferal Research)
7. Sujata Kurtarkar, R. Nigam, R. Saraswat & **Linshy V. N.,** Response of benthic foraminifera *Rosalina leei* to factors associated with salinity changes: A laboratory culture study *(Communicated)*.
8. Sujata R. Kurtarkar, **Linshy V. N.**, R. Saraswat & R. Nigam Response of *Rosalina* sp. to different temperatures and salinities : A laboratory culture experiment. *(Communicated)*.
9. Sujata Kurtarkar, R. Nigam, **Linshy V. N.** & R. Saraswat, Effect of temperature and salinity on stable isotopic composition of *Pararoptalia nipponica* and *Rosalina* sp.: A laboratory culture experiment *(Communicated)*

Abstracts

1. **Linshy V.N.**, Sujata R. Kurtarkar & R. Nigam (2005) Lifespan of smaller benthic foraminifera *Strebloides* sp. as observed in laboratory culture experiment, *in*: Abstract Volume, XXth Indian Colloquium of Micropaleontology and Stratigraphy, Visakhapatnam, India, p 54.

2. Nigam, R., Sujata R. Kurtarkar, Saraswat, R., **Linshy V.N.** & Rana, S.S. (2005) Response of benthic foraminifers *Rosalina leei* to different temperature and salinity, under laboratory culture experiment, *in*: Abstract Volume, XXth Indian Colloquium of Micropaleontology and Stratigraphy, Visakhapatnam, India, p. 72.

3. **Linshy V.N.**, Sujata R. Kurtarkar, Nigam, R. & Kavita, M. (2007) Application of benthic foraminifera in monitoring marine pollution: effectiveness of laboratory experiments, *in*: Abstract Volume, XXIth Indian Colloquium of Micropalaeontology and Stratigraphy, Birbal Sahni Institute of Palaeobotany, Lucknow, p.101

4. Sujata R. Kurtarkar, **Linshy V.N.**, & R. Nigam (2007) Effect of different combinations of salinity and temperature on benthic foraminifera *Pararotalia nipponica*: observations from laboratory culture experiment, *in*: Abstract Volume, XXIth Indian Colloquium of Micropalaeontology and Stratigraphy, Birbal Sahni Institute of Palaeobotany, Lucknow. India. p.100

5. **Linshy V.N.**, Sujata R. Kurtarkar & Nigam, R. (2007) Ecological studies on benthic foraminifera: glimpses from laboratory experiments, *in*: Abstract Volume, 44th Annual convention and meeting on Science of shallow subsurface, Indian Geophysical Union at Kurukshetra University, Haryana, India,

6. Nigam, R., **Linshy V.N.**, Sujata R. Kurtarkar (2008) Experimental studies on benthic foraminifera: The Indian Perspective, *in*: Abstract Volume, The fifth International conference on Environmental Micropalaeontology, Microbiology & Meiobenthology, EMMM 2008, University of Madras, India, p.227.

7. Sujata R. Kurtarkar, **Linshy V.N.**, Nigam, R. (2008) Response of *Pararotalia nipponica* to variations in temperature-salinity in laboratory experiments: Implications in climatic reconstruction, *in*: Abstract Volume, The 5th International conference on Environmental Micropalaeontology, Microbiology & Meiobenthology, EMMM 2008, University of Madras, India, p.173.

8. **Linshy V.N.**, Sujata, R. Kurtarkar & Nigam, R., (2008) Benthic foraminifera as bio indicators of marine pollution, *in*: Abstract Volume, The Micropalaeontological Society's Foraminifera and Nannofossil Group's joint Spring meeting 15-17th May, University of Tubingen, Germany.

9. Sujata, R.K., Nigam, R., Saraswat, R., **Linshy, V.N.** (2008) Regenerative capability of benthic foraminifera *Rosalina leei, in*: Abstract Volume, 45th Annual convention and meeting of Indian Geophysical Union 5-7 November, Banaras Hindu University, Varanasi, U.P. India, p.70.

10. Sujata R. Kurtarkar, **Linshy V.N.**, Saraswat, R. & Nigam, R. (2009). Laboratory culture experiment to study the effect of temperature and salinity on growth and reproduction of benthic foraminifera *Rosalina* Sp. *in*: Abstract volume of Indian Geophysical Union, the 46th Annual convention and meeting on Evolution of Himalayan foreland basin and emerging exploration challenges. Wadia institute of Himalayan geology, Dehradun, India, p. 21.

11. Sujata R. Kurtarkar, **Linshy V.N.**, Saraswat, R. & Nigam, R. (2009) "Laboratory culture experiment on benthic foraminifera *Cymbaloporetta plana* (Cushman, 1924); Role of temperature and food on growth and reproduction *in*: Abstract volume of XXII Indian Colloquium of Micropalaeontology and Stratigraphy, National college, Thiruchirapally, Tamilnadu, p.116.

12. Sujata R. Kurtarkar, R. Nigam, **Linshy, V.N.**, R. Saraswat (2010) Changes in the stable oxygen isotopic composition of Pararotalia nipponica and Rosalina with reference to temperature and salinity: Culture experiments. 2010 Ocean Science Meeting to be held at Portland, Oregon, USA, 22nd to 26th February.

Annexure 2

Translation
Document of Award

The Deutscher Akademischer Austauschdienst (German Academic Exchange Service) is a joint organization of the universities and other institutions of higher education in the Federal Republic of Germany. Supported from public funds, the DAAD promotes international academic cooperation, especially through the exchange of students and academics. DAAD scholarships are awarded by selection committees comprising a panel of independent academics.

The person named in the overleaf 'Stipendienurkunde' has been awarded a scholarship by the DAAD for further academic study and training in Germany.

I would like to congratulate you on your award and wish you a successful stay in Germany. Besides working in your special academic field, I hope you will make use of the opportunity to get to know our country, its people and its culture better. It would give me great pleasure, if – after returning to your home country – you would maintain contact with your German partners and the DAAD.

Prof. Dr. Stefan Hormuth
President of the DAAD

DAAD Deutscher Akademischer Austausch Dienst
German Academic Exchange Service

STIPENDIENURKUNDE

Der Deutsche Akademische Austauschdienst ist eine gemeinsame Einrichtung der deutschen Hochschulen.
Er fördert mit öffentlichen Mitteln die internationale akademische Zusammenarbeit, insbesondere den Austausch von Studierenden und Wissenschaftlern.
Die Stipendien des DAAD werden auf der Grundlage von Auswahlentscheidungen unabhängiger wissenschaftlicher Kommissionen vergeben.

Im Rahmen seiner Programme verleiht der Deutsche Akademische Austauschdienst

Linshy Valiyaparambil Nanappan

ein Stipendium zur wissenschaftlichen Aus- und Fortbildung in Deutschland.

Ich beglückwünsche Sie zu diesem Stipendium und wünsche Ihnen einen erfolgreichen Aufenthalt in Deutschland. Ich hoffe, dass Sie neben Ihren fachlichen Aufgaben auch die Gelegenheit wahrnehmen werden, unser Land, seine Menschen und seine Kultur näher kennenzulernen. Ich würde mich freuen, wenn Sie auch nach Rückkehr in Ihr Heimatland weiterhin die Verbindung mit Ihren deutschen Partnern und dem DAAD aufrechterhalten würden.

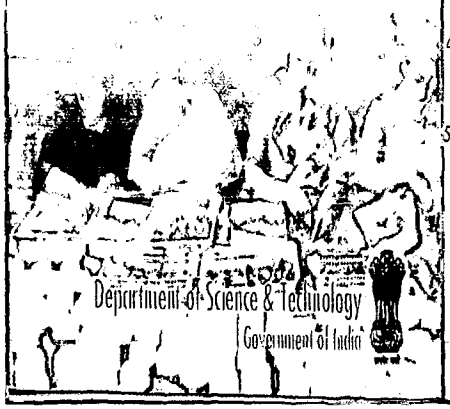
Bonn, den 11.03.2008

Deutscher Akademischer Austausch Dienst DAAD

Prof. Dr. Stefan Hormuth
Präsident des Deutschen Akademischen Austauschdienstes

Science & Technology ForWomen

FELLOWSHIP SCHEME FOR WOMEN SCIENTISTS FOR SOCIETAL PROGRAMMES



No. SSD/SS/006/2004
Government of India
Department of Science and Technology
Science & Society Division

Technology Bhavan
New Mehrauli Road
New Delhi - 110 016.

Dated the March 15, 2005

ORDER

Sub: Financial assistance for the project entitled "Development of advanced technique for effective monitoring of spatial and temporal variation in marine pollution through laboratory culture of formanifera." under the guidance of Mrs. Linshcy V.N., Micropalcontology Lab., Geological Oceanography Division, National Institute of Oceanography, Dona Paula-403004, Goa.

Sir

Sanction of the President is conveyed to the approval of the project at a total cost of Rs.4,84,885.30/- (Rupees four lakhs eighty four thousand eight hundred eighty five thirty paise only) for duration of two years.

2. The items of expenditure for which total allocation of Rs.4,84,885.30/- has been approved are given below:

	Heads	1st Year	2nd Year
I.	**Manpower** Scholarship to Scientist (@ Rs.10,000/- p.m.)	1,20,000/-	1,20,000/-
II.	**Consumables**	50,000/-	30,000/-
III.	**Contingency**	10,000/-	10,000/-
IV.	**Travel**	15,000/-	15,000/-
V.	**Equipment** (Stereo zoom binocular microscope)	74,885.30/-	--
VI.	**Overheads**	25,000/-	15,000/-
	Total	2,94,885.30/-	1,90,000/-

GRAND TOTAL : Rs.4,84,885.30/-

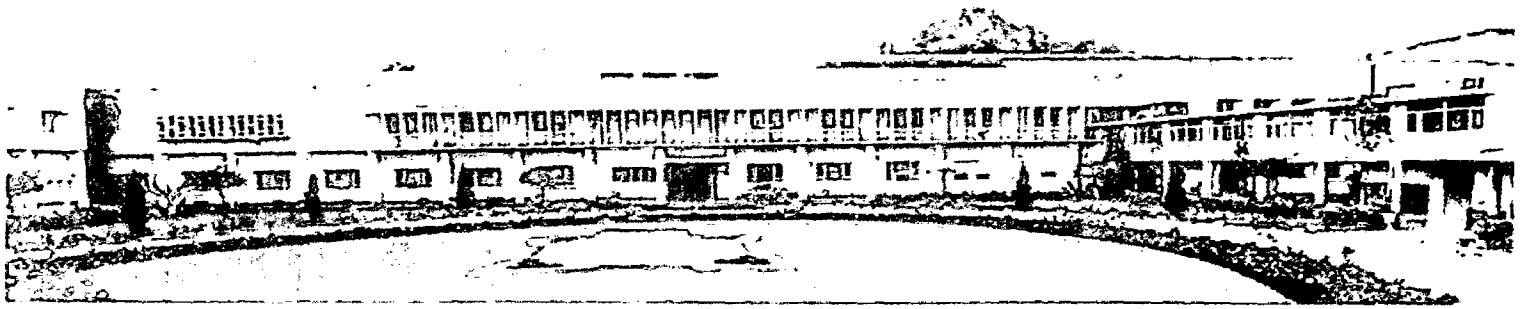
3. Sanction of the President is also accorded to the payment of Rs.2,70,000/- (Rupees two lakhs seventy thousand only) to Geological Oceanography Division, National Institute of Oceanography, Dona Paula-403004, Goa being the first installment of grant for implementation of the said project during 2004-2005.

4. This is being a new project to this organization, there is no question of getting utilization certificate for the earlier grant.

5. The expenditure involved will be debitable to Demand No.83

Major Head 3425 - Other Scientific Research

Annexure 4



XXI Indian Colloquium on Micropalaeontology and Stratigraphy

(November 16-17, 2007)

Certificate

organized at

Birbal Sahni Institute of Palaeobotany, Lucknow

This is to certify that the poster presentation by

Sinshy K.N., Sujata R. & Kurtarkar, R. Nigam & M. Kavita

has been awarded Best Poster Prize

Rahul Garg

(Rahul Garg)
Convener