Running title: FORAMINIFERA: DEAD OR ALIVE?

MORTALITY, PROTOPLASM DECAY RATE, AND RELIABILITY OF STAINING TECHNIQUES TO RECOGNIZE 'LIVING' FORAMINIFERA

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ABSTRACT

Non-vital staining, especially with rose Bengal, has been widely used in ecological studies to differentiate between the tests of dead(unstained) foraminifera from those presumed to be living at the time of collection (stained). Doubts have been expressed about staining methods because of the possibility that dead individuals may retain undecayed protoplasm for weeks or months after death; when stained, such individuals would be recorded as living. To assess the importance of such false positives, it is necessary to examine rates of mortality, and the modes of generation of empty tests, i.e., whether due to reproduction, growth stages (leaving empty tests during growth) or death. It can be argued that reproduction, ontogeny, and death through predation lead to tests devoid of protoplasm. Whereas reproduction may affect only a small proportion of the population of each species (due to high pre-reproductive mortality), predation in oxygenated environments may be responsible for the major part of that pre-reproductive mortality. In oxygenated environments, disease or adverse environmental conditions are most likely to lead to dead individuals having tests containing protoplasm. In dysaerobic/anoxic environments, predation by macrofauna may be excluded, so foraminifera die through other causes and thus more tests with dead protoplasm may be potentially available for staining. Therefore, for most other environments, the problem of staining dead individuals is almost certainly overstated. Furthermore, from comparative studies, it seems that the most commonly used technique (staining with rose Bengal) is as reliable as others. Now that new vital staining techniques, especially the use of fluorescent probes, are being introduced, it is timely for further objective comparative studies of all techniques to be made in order to evaluate data already gathered and to develop the best strategies for future ecological studies according to whether they are field-based or experimental.

INTRODUCTION

Ever since Walton (1952) introduced the rose Bengal staining method to distinguish live foraminifera from dead ones, it has been widely used in field-based ecological studies. Subsequently, other methods have been introduced. In a comprehensive review, Bernhard (in press) divided them into 'terminal' (causing death) and 'non-terminal' (not causing death). Terminal methods include the use of nonvital stains (rose Bengal, Sudan Black B), an adenosine triphosphate assay (ATP), as well as ultrastructural observation and *in situ* embedding techniques (Bernhard and Bowser, 1996). Non-terminal methods include vital staining (e.g., with fluorescent probes), direct observation (protoplasmic color, apertural bolus, cytoplasmic streaming, reticulopodial networks), negative geotaxis, or density-gradient centrifugation. Bernhard discussed the advantages and disadvantages of each method and concluded that, to be confident of results, more than one (preferably disparate) method be applied.

From a practical point of view, different types of study require different methodologies. It is obviously important to choose the method appropriate to the type of study being undertaken. Field-based studies involving distribution and quantification of foraminiferal assemblages require techniques which can be used on preserved samples,since it is not practical to examine a large number of samples immediately after collection. Also, samples containing a high proportion of silt and mud sized material have to be sieved to concentrate the foraminifera and this may harm forms that are still alive. For these kinds of samples, the most appropriate method is staining and this is why rose Bengal has been routinely used. By contrast, experimental studies require separation of

live individuals without harming them and this is most readily accomplished by the new fluorescence techniques. Furthermore, it may be necessary to test whether individuals are still alive after a few days hence Berhard's recommendation that two disparate methods be used to evaluate this (e.g., Alve and Bernhard, 1995; Moodley and others, 1997). Field-based and experimental studies give complementary data on foraminiferal ecology. They are not mutually exclusive alternatives; both are necessary approaches to solving ecological problems.

One criticism of some terminal techniques (especially nonvital staining) is that not only living foraminifera will stain, but also dead ones where protoplasm has not yet decayed completely (e.g., Walker and others, 1974; Bernhard, 1988). However, such studies did not consider the processes leading to the contribution of dead individuals to the sediment or whether their tests would contain protoplasm. The aim of this paper is to evaluate the available information on the processes of mortality, the likelihood of there being dead foraminiferal tests containing protoplasm, and the rate of decay of protoplasm in the natural environment. These have particular relevance to assessing the validity of data obtained from past field studies based on nonvital staining.

MORTALITY AND SURVIVORSHIP

Mortality refers to death; survivorship is the opposite. For example, a species population which has undergone 60 % mortality has 40 % survivors. On theoretical grounds, a range of patterns of mortality and survivorship can be recognized, the extremes being 1) high infant mortality followed by low adult mortality and 2) low mortality of infants and adults followed by high mortality in old age.

Because foraminifera typically produce many offspring during each reproductive event, it is commonly assumed that they have experienced high infant mortality as a counterbalance. However, this clearly does not always apply because, as many are r-strategists, they can respond rapidly to increased food availability by increasing their numbers (higher rate of survivorship). In reality, there are few data on mortality in foraminifera and these are based on assumptions concerning growth rate. For instance, Hallock and others (1986) calculated that 88% of *Archaias angulatus* died in the first 9 months, prior to the onset of reproduction in months 9-10. Adults that reproduced had a diameter >2200 μm and forms <1000 μm in diameter (here considered as juveniles) showed 58% mortality.

GENERATION OF EMPTY TESTS

Empty tests can result from reproduction, growth stages, and death.

REPRODUCTION

During reproduction, the parent organism divides into either agamonts (asexual) or gametes (sexual), usually leaving the original test devoid of protoplasm. Very rarely, a small residue of protoplasm may be left (Lee and others, 1991; Goldstein and Moodley, 1993). In the case of *A. angulatus* discussed above, 88% of individuals died prior to the onset of reproduction so not more than 12% 'died' as a consequence of reproduction.

Growth Stages

A source of empty tests, due to growth, is found in globular and flask-shaped unilocular forms. Such tests (regardless of wall type) cannot easily be increased in size and Murray (1976) suggested that species such as *Saccammina atlantica* would leave the test and grow a new, larger one. Indeed, it was inferred that *S. atlantica* might have four growth stages, leading to a skewed contribution to the "dead" assemblage This concept has subsequently been shown to be true for *Astrammina rara*, where one individual in culture abandoned nine tests, each lacking cytoplasm (Bowser and others, 1995).

Pre-reproductive Death

How do foraminifera die? Possible causes include being eaten, starvation, disease, and naturally adverse environmental conditions but there are few data on any of these. Perhaps the most likely cause of death is being eaten, since the meiofauna (Giere, 1993), including foraminifera, are potential food for a variety of organisms (see summaries by Murray, 1991, pp. 13-14; Gooday and others, 1996). Calcareous tests survive passage through the digestive tract of predators and are contributed to the sediment (Myers, 1943; Boltovskoy and Zapata, 1980; Palmer, 1988). A series of experiments designed to assess the impact of predation in the Indian River, Florida, USA, showed standing crop values (based on stained assemblages) five to six times higher in screened cages (which excluded predators >1 mm) than outside the cages where predation took place (Buzas, 1982). Assuming zero predation inside the screened cages (and no significant increase in deaths due to competition for food), this means that around 80% of the mortality of foraminifera in the Indian

River can be attributed to predation. Similar experiments carried out in a water depth of 125 m also showed significantly increased densities in the cages screened from predators (Buzas and others, 1989). Palmer (1988) showed that fish predation on foraminifera reduced their numbers by 19-31% during experiments in a flume, with or without water flow. Even though these figures should not be directly extrapolated to other environments, they suggest that predation is significant and possibly the main cause of pre-reproductive death.

IMPLICATIONS

Two important points arise: death resulting from reproduction, growth stages, and predation leads to tests already devoid of protoplasm. Likewise, any individuals that die through starvation probably have tests that contain very little protoplasm. The only modes of death that are likely to leave tests containing significant amounts of protoplasm are disease and adverse environmental conditions (such as anoxia) and, at present, there are no quantitative data on either of these. It follows that under natural conditions there may not be large numbers of dead foraminiferal tests with decaying protoplasm.

DECAY

Following death, decay leads ultimately to the remineralization of the organic material. Presumably once the cell membrane breaks, the cell contents may be more readily degraded and dispersed. The process is initiated by autolysis - the operation of lysosomal enzymes present in the cell. For the use of a nonvital stain, the key point of interest is how long it takes before the

protoplasm disperses as an organic soup that no longer takes up stain. Thus, there are two fundamental questions:

How long does it take for foraminiferal protoplasm to decay after death?

How reliable are staining methods for distinguishing live from dead individuals?

These two questions are both separate and related. If the rate of decay is such that protoplasm is destroyed to a non-staining state within hours or perhaps a few days, then it is reasonable to assume that any test with protoplasm is either a living or a very recently dead individual. Nonvital stains can then be used to make it easier to distinguish between 'live' and dead individuals. However, if the rate of decay is so slow that stainable protoplasm persists for several weeks or months after death, then non-vital staining will not satisfactorily distinguish between dead and living individuals and therefore will give unreliable results. In a study of Antarctic foraminifera Bernhard (1993) made ultrastructural studies of selected individuals. In one case, the individual was dead. The protoplasm had decomposed but she could not determine how long it was since death. Goldstein and Corliss (19994) made ultrastructural studies on foraminifera (which appeared to contain protoplasm) collected from 710 m and found that few were alive at the time of fixation. It is not known to what extent the rapid pressure change from 710 m (71 atmospheres) to one atmosphere affects living foraminifera. Similarly, material collected from >500 m from the Gulf of Mexico also contained degraded protoplasm but the authors could not determine either the time of death or the rate of decay (Sen Gupta and others, 1997). Clearly, these test contents would stain with rose Bengal to give false positives.

How Long does it take for Foraminiferal Protoplasm to Decay After Death?

From a theoretical point of view it would be expected that the rate of autolytic or bacterially-mediated decay would depend on water temperature (faster at higher temperatures) and oxygen concentration (faster at higher oxygen levels). Apart from decay, the protoplasm is a potential food resource for other microorganisms. Indeed, in cultures of *Elphidium crispum* monitored daily for movement, dead individuals (i.e., those that had ceased to move) soon become surrounded by a halo of ciliates and flagellates which were presumably feeding on the food resource (unpublished observation, JWM). Immediately after gamete release in *Ammonia beccarii* predatory dinoflagellates and ciliates enter the test and feed on residual cytoplasm and unreleased gametes (Goldstein and Moodley, 1993). *Allogromia* (strain NF) harbors bacteria on its test (Pierce and Nathanson, 1974) which, following death, might degrade the protoplasm and organic wall.

There is great uncertainty about how long protoplasm takes to decay but Bernhard (1993) noted that 'it is known that ultrastructural changes (e.g., mitochondrial swelling, plasma membrane rupture) appear within hours to days in other cells'. Experiments aimed to address this question are complicated because the mode of killing the foraminifera will inevitably be different from natural mortality and therefore might prejudice the results. Boltovskoy and Lena (1970) killed benthic foraminifera by placing them in an oven at 70°C for half an hour. They then placed the specimens in ambient seawater at 25-35°C. They took samples at regular intervals, stained them with rose Bengal, and determined that the protoplasm had all decayed by the end of the 65 day experiment. They repeated the experiment but used distilled water rather than heat as the method of killing. The dead foraminifera were then kept at 15-20°C and after 60 days some specimens still contained protoplasm that stained with rose Bengal. Bernhard (1988) showed that protoplasm remains for at least 4 weeks in Antarctic foraminifera (ambient temperature 1°C) killed by raising the temperature

to 20°C for 1 hr. However, it is not clear that degrading bacteria survived the heat treatment so the results may be artificial. Indeed, all these experimental results must be treated with caution because of the artificial manner of death and because in none of them were the specimens subsequently kept in a natural environment with all the other associated micro- and meiofauna, including predators.

Corliss and Emerson (1990) speculated that organic carbon (presumably mainly non-labile/refractory) in deep-sea sediments may be degraded over a period of a few months to several years, but this has no relevance to the decay of foraminiferal protoplasm (where the organic carbon is labile) although they linked the two. Hannah and Rogerson (1997) tested the rate of decay of cytoplasm under anoxic conditions by degassing sediment with N_2 and storing it at 10°C in the dark. After 5 days, only 10% of the foraminifera were active. In these anoxic conditions, even after three months, dead individuals retained protoplasm which stained with rose Bengal, as would be expected with a nonvital stain. Under natural low oxygen conditions where foraminifera are able to survive but the animals which prey on them can not, individuals will die through other causes. This has two consequences: more foraminifera will survive, and they will then die through causes that will leave dead protoplasm-filled tests that may become stained. However, this unusual situation -- specific to dysaerobic and anoxic settings where predators are largely excluded -- should not be taken as applicable to oxygenated environments or to dysaerobic sediments sitting beneath an oxic surface layer where predators are not excluded. The processes of bacterially mediated decay operate in both oxic and anoxic settings and the quantitative efficiency with which aerobic and anaerobic bacteria degrade organic matter is essentially the same (Tyson, 1987). It does not follow that the rates of decay are everywhere the same, so observations from any one area or environment may not be globally applicable.

Comments on Techniques to Differentiate Live and Dead Foraminifera

Certain fluorescent probe techniques are powerful methods for identifying living individuals for experimental work and observation of life processes (Bernhard and others, 1995; Bowser and Bernhard, in prep.). Of course, with the use of any non-terminal technique, care must be taken to ensure that individuals survive sampling and incubation in the probe without being killed (for instance, dying from elevated temperatures). Tests occupied by living cryptic or hermit organisms will yield a positive result whether the ATP, vital, or nonvital staining technique is used. Therefore, none of these methods has an advantage over the others in this respect.

The ATP method is expensive both in time (e.g., becoming competent and to carry out the analyses, particularly those samples with a low live-dead ratio) and in the provision of equipment. Like non-terminal techniques, it may also require special handling of the samples in order to keep the foraminifera alive and unmodified between the time of collection and the time of sample preparation. By contrast, nonvital staining can be carried out on preserved samples a long while after they are collected, the procedure is simple, takes very little time, and is inexpensive. For those sediments where detrital particles far outnumber foraminiferal tests, there is the great advantage that the stained sample can be dried and foraminifera concentrated using a heavy liquid such as either carbon tetrachloride or trichloroethylene. Even though drying leads to loss of soft-bodied forms, this is not necessarily important if the ecological studies are to provide analogs for palecocology since such forms would not be preserved in the fossil record except under very unusual conditions.

A comparison of ATP and rose Bengal staining was made by Bernhard (1988), who concluded that more tests were stained than predicted to be alive by ATP analysis. Experimental studies by Linke and others (1995) have shown that the amount of ATP present in *Cribrostomoides subglobosus* is related to feeding activity. In the deep sea, food arrives in pulses and this species responds by rapidly feeding. In the experiments, prior to food uptake ATP levels were low, but three days after feeding they increased considerably and after a further three days reverted to their former levels. Thus the amount of ATP present in an individual is related to its recent feeding activity. However, in experimental studies, Alve and Bernhard (1995) concluded that rose Bengal staining gave more reliable results than ATP analysis. Bernhard and others (1997) also compared the results of rose Bengal staining of sediment samples with a limited sample of fluorescently-labelled foraminifera in the surface 1mm of a core from an oxygen-deficient environment and found the numbers to be 'roughly comparable'. This suggests that, in this instance, the two techniques have similar reliability. Lutze and Altenbach (1991) compared their data on material stained with rose Bengal with analysis of organic carbon. They concluded that if the rose Bengal method is used with care it gives results which are 96% correct. From these various comparisons it may be concluded that results obtained by using rose Bengal to differentiate foraminifera living at the time of collection are as reliable as those obtained by other techniques. This is important because rose Bengal staining has been by far the most commonly used technique for distinguishing between living and dead individuals in field-based studies.

CONCLUSIONS

From a consideration of life processes of foraminifera, it has been argued that the majority of tests contributed to the sediment are emptied of protoplasm as a consequence of reproduction, growth stages, or predation. Therefore these tests will not stain to give false positives. Only death through disease or adverse environmental change are likely to lead to the presence of tests containing dead protoplasm. Notwithstanding limited experimental data which has promoted the view that foraminiferal protoplasm decays slowly, there is strong circumstantial evidence that the protoplasm decays quickly as in other meiofauna. Nevertheless, the differentiation of living and dead individuals continues to be an essential requirement for ecological studies. Each technique has its merits but also its disadvantages. There is as yet no universally-applicable technique, and all represent a compromise between costs (in terms of materials, equipment, and especially of time to perform) and reliability. An awareness of the strengths and limitations is essential for whichever technique is used. Where comparisons have been made between pairs of techniques, in general the results have been quite similar, and that is an encouraging outcome. The rose Bengal staining method has the most widely used to date for field-based studies and it may be concluded that results obtained are as reliable as those by other techniques. However, with the introduction of new techniques, such as the use of fluorescent probes, which are clearly ideal for use in experimental studies, it is timely for further comparative studies to be made not only of all the different techniques but also their use in a wide range of environmental settings (cold/warm, oxic/dysaerobic/anoxic, brackish/marine/hypersaline). Such studies will be important both to evaluate the data of past field-based studies and for planning methodologies to be used in future field-based and experimental ecological studies.

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