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Biogeosciences Discussions

Interactive comment on "Determination of the metabolically active fraction of benthic foraminifera by means of Fluorescent in situ Hybridization (FISH)" *by* C. Borrelli et al.

Anonymous Referee #2

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The topic of this paper should be of interest to a number of foraminiferal ecologists, which are probably not a major portion of the Biogeosciences audience. In that regard, it is not obvious that this journal is the best possible journal for this contribution.

As for the approach, the rationale is adequate: there are no perfect methods to differentiate live and dead foraminifera. This manuscript, however, oversimplifies this new method and does not fully explain the negative aspects of such an approach. Below are listed a number of issues that must be addressed by these authors before this paper can be published in any reputable journal. Please note that the majority of this review was written prior to the posting of Dr. de Nooijer's comments. In some cases,

the following points reiterate those made by de Nooijer and, in general, I agree with his assessment.

1) How did the authors distinguish between the dim fluorescence in (1) “washed shells” (page 7483 line 20, Figure 2H), (2) specimens exposed to nonsense probes (Figure 3B), and (3) starved (but living) individuals (Figure 5)? This is a major problem with this approach.

2) The contribution relies on extremely low numbers where there is no statistical power whatsoever. In addition, for example, the efficacy of this approach was only 50% for Eggerella (page 7485 line 5). It is impossible to say what the efficacy of the method was for the other species given the text is not possible to understand (see also next point).

3) Some sections of the text are un-interpretable (e.g., page 7484, Line 15-22; Table 3). Of these 19 Ammonia, how many were living? Although the text states that 17 emitted fluorescence, the text does not say if they were live or dead as determined by optical means. The same question arises for the other 7 that were incubated with S17. Thus, it is impossible to know what the efficacy of this method was. Also, Table 1 appears to show that the FISH approach overestimates the proportion of living specimens (but, frankly, the table is un-interpretable).

4) What is the bulge in the upper right in Figure 4B? It does not appear in the “optical” image (A), and why is there another fluorescent object to the right of the foraminifer? Please clarify why these label with the FISH probe.

5) Specimens were air dried (page 7481, line 20), so why does the fluorescent signal appear to include the entire volume of the shell (see Figures 4-5)? When dried, foraminiferal cytoplasm will not fill all chambers of their shells (cytoplasm is mostly water, and thus when dried, it will only fill a small fraction of the shell). Along this line of argument, it is extremely peculiar that the fluorescence was homogeneous in the protoplasm given these specimens were dried (page 7485, line 9-15). Imagine

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a grape versus a raisin. It is impossible that dried cytoplasm would have remained homogeneously distributed in dried shells. Thus, the fluorescence can not represent foraminiferal cytoplasm. Plus, to test false positives of recently dead specimens (page 7482 line 17), why were specimens dried for an hour when air drying was a step used in the actual FISH protocol (page 7481 line20)?

6) Were all specimens examined at the same magnification? Given that fluorescence brightness is exponentially proportional to magnification, it is important to use identical magnification in all cases.

7) The means used to optically identify living specimens are not all inclusive: Pseudopods are not always deployed by living individuals and some foraminifera do not consume algae (thus color can not be used as an indicator of health).

8) In at least some environmental settings, rRNA can be preserved for considerable periods after an organism's death (e.g., Coolen and Overman 2007 Environmental Microbiology; Panieri et al. 2010 Geobiology). The authors need to address this potential complication.

9) How useful will this method be for foraminiferal workers in general, given that sometimes samples can not possibly be analyzed within one week (line 18 page 7481)?

10) How can this method be used for deep-sea species that undergo huge pressure and temperature changes upon collection? Surely these foraminifera will die upon collection, and thus would give negative results when it is certain that they were living in the deep sea.

11) The Discussion grossly overstates the reliability and realities of the method (it is not cheap [reagents are expensive, the required microscope is expensive], it is not easy [there are many potential pitfalls for FISH on any taxon], and not quick [it still takes time to pick the specimens and then do an overnight preparation with many hybridization steps]!). Detailed comments on this problem appear among other issues listed below.

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Page 7475 Title: The title is misleading because the specimens subject to some of these protocols have been killed, thus can not be metabolically active. The work was done while Borelli was in Italy; she recently moved to USA. Thus the USA address should be noted as “current address”, not her primary address.

Page 7477 The opening statement (lines 2-4) is not a sentence. Line 4: please define “hard shelled”. Line 15: Murray and Bowser (2000) did not address the fate of “animal” tissue (Foraminifera are not animals). Further, Murray and Bowser is a theory paper, not a paper that actually demonstrated these results.

Page 7478 Line 3 states that CTG is time consuming, implying that the FISH method is not. This is not true: FISH takes just as much time, if not more, than CTG. Line 5: omit “s” on “controls”. Line 9: FISH is not “novel”; it has been in existence for at least a decade. Line 15: add “of” between “study” and “prokaryotes”. “Prokaryote” is an antiquated term (see Pace, 2006 Nature). The stated goal (lines 28-29) that FISH is “more efficient, simple and rapid... than other available methods” is not addressed truthfully in the Discussion. Further, this assertion is highly debatable. The authors need to discuss the advantages and disadvantages of the method in the Discussion section (see above and below). FISH is not simple, not rapid, and not more efficient.

Page 7479: What was the water depth where the samples were obtained? Line 5-6: How do the authors know the top 2 cm contained the highest abundance of foraminifera? What was the temperature at the field sites? How much did temperature change during the 2-4 hours of transport to the laboratory? What was the salinity, pH and carbonate chemistry of the sites? Line 15: In microbiological terms, “culture” indicates reproduction (or division). This is not the case in this contribution and thus this term should be stricken from this contribution (also line 1 page 7480, etc). Line 19: How did the populations obtain oxygen? How might decreased oxygen availability affect results?

Page 7480 Line 6: The authors likely intend to use the term “assemblage” rather than

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"association". Line 17: Given the algal food offered to the foraminifera (Dunaliella and Chlorella, which are green), it is strange that yellow and light brown were used as distinguishing color characteristics. The specimens should have been bright green (see, for example, Barras et al., 2009 J Foraminiferal Res). Line 21: Why were Ammonia and Miliolidae selected?

Page 7481 Line 9-10: Photosynthetic pigments always autofluoresce at proper excitation / emission wavelengths. If this was not observed then the authors were doing something wrong microscopically and/or the specimens had not ingested algae. Perhaps the authors intend to note that pigment autofluorescence does not exist at the wavelengths they used. Line 20: As noted above, specimens were air dried, so why does the fluorescent signal appear to include the entire volume of the shell (see Figures 4-5)? The foraminiferal cytoplasm, when dried, will not fill all chambers of the shells (cytoplasm is mostly water, and thus when dried, it will only fill a small fraction of the shell). The methods section (page 7481-7482) is far too detailed. It reads like a term paper or thesis.

Page 7482 Line 12: Omit second "s" from "Specimens" Line 17: To test false positives of recently dead specimens, why were specimens dried for an hour when air drying was a step used in the actual FISH protocol (page 7481 line20)? Line 20-21: It is unclear why SEM was used given fluorescence is not detected with SEM.

Page 7483 Line 1: "e" should be "y" in "microscope". Line 4-7: Why weren't the same measurements done on live vs dead specimens? Why was this only done on fed versus starved populations?

Page 7484 Line 4-7: Did doubling the time in NaClO make any difference? Why is this data not shown? It appears that the authors merely assumed it made a difference. Line 9: Please define where coastal sediments were obtained (are these equal to Falconara and Portnovo?) Line 10: Most people would call FISH a probe that hybridizes, it is not a "stain". Line 11-15: This repeats what is stated in the Methods, it should be omitted

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here. Line 13: Proper spelling is “species” (there is no such thing as a “specie”). Page 7484, Line 15-22: As noted above, this section is un-interpretable. Table 3 is also not clear. Of these 19 Ammonia, how many were living? Although the text states that 17 emitted fluorescence, the text does not say if they were live or dead as determined by optical means. The same question arises for the other 7 that were incubated with S17. Thus, it is impossible to know the efficacy of this method.

Page 7485 Line 3: Perhaps the authors intend to state “...agglutinated particles in the shell...”? Line 9-15: As noted, it is extremely peculiar that the fluorescence was homogeneous in the “protoplasm” given these specimens were dried. Imagine a grape vs a raisin. It is impossible that dried cytoplasm would have remained homogeneously distributed in dried specimens. Thus, the fluorescence can not represent foraminiferal cytoplasm. Line 22-23 requires a literature citation. Line 27: It is unfathomable that this part of the study relied on 3 specimens. Perhaps the authors intend to state that pooled samples were analyzed? If so, how many specimens per sample? This point requires distinct clarification.

Page 7486 Line 13 and 15 should state the number of individuals rather than percentages. In reality, only 5-6 specimens were living when using color and pseudopodial extension as criteria and only 4-5 fluoresced. These are such small numbers that this entire paper is flawed. Line 22: Bernhard (2000) discusses terminal versus non-terminal methods.

Page 7487 Somewhere near line 2 the authors need to state that their method is a terminal method, and thus their arguments should have no impact on non-terminal method assessments. Also, it must be noted that one of the major advantages of CellTracker Green method is that it can be used as a non-terminal method. Line 7: The sentence should end in “... as a live-dead indicator.” Line 8: “were” should read “was”. Line 14-17: The qualifier “non-fixed” needs to be included in this statement because it is established that chemical fixation with formalin or other aldehydes causes some autofluorescence at Cy3 wavelengths. Line 19: “(i.e., the intracellular...” must

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be changed to "(i.e., our intracellular. . ." because TEM distinguishes unequivocally live from dead and this intracellular method should not be considered equivalent to the author's "intracellular analysis" by merely assessing color. Line 17-19: The meaning of this statement is unclear. The authors thus need to clarify it. It is impossible to comment on its content. Line 28: This sentence needs to cite Bernhard et al. (2006) which showed this. Thus, the statement should read ". . .marine sediments, as noted by Bernhard et al. (2006)." Line 29: "the use of the term "reliable" is unacceptable. This method has not been shown to be reliable, given the extremely small population sizes and severely limited species composition.

Page 7488 Line 7: "die, his cellular" should read "dies, its cellular" (foraminifera do not have gender). Line 9-23: The assertion that this method is "reliable" is unfounded, the assertion that the method is "much less time consuming" is also unfounded since FISH takes quite a bit of time. Specimens still need to be picked and the lab work is intensive. Plus, an epifluorescence microscope, which is not inexpensive, is also required for this method. The authors need to discuss exactly how much time and cost is involved with this method and how long it took them to actually get it to work. Not all species should be expected to respond the same way, so each laboratory will have to repeat the same pitfalls. This paper presents the method as "easy", which is an overstatement. And, the method certainly should not be reported to work for all foraminifera, given only about 4 species have been tested. There are many species with opaque shells. Plus, what about for deep sea species that undergo huge pressure and temperature changes upon collection? Surely they will die upon collection, and thus would give negative results when it is certain that they were living in the deep sea.

Page 7489: This entire page needs to be rewritten in light of comments throughout this review.

The manuscript would benefit greatly from the expertise of a native-English speaking individual. See, for example, page 7483 lines 13-25m which are particularly awkward and undecipherable.

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Table 1: What does “NO” mean in the column headed “Autofluorescence assay”? Does NO = nitrous oxide? Does NO= no (if so, what does that mean exactly? The assay was not done? Why have this column if all species were treated the same way?)

Table 3: Define the difference between zero and “none”?

Figure 2: What does “particular” mean in the caption for B and F? Figure 3 caption should read “Little fluorescence occurs” instead of “No fluorescence occurs” given that the specimen can be seen with epifluorescence.

Interactive comment on Biogeosciences Discuss., 7, 7475, 2010.