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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.

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Dear Dr. Middelburg,

For responding to the reviewers' comments, we would like first to provide a brief description of the FISH technique and explain the rationale behind its application. We apologize for not having illustrated better these issues in a previous version of the manuscript. We hope that this will allow to better understand the advantages of this technique, when compared with other methods currently utilized for quantifying live and active foraminifera. As stated by Referee #2, there are no perfect methods for differentiating between live and dead foraminifera, and all

the available methods suffer from advantages and limitations. In this regard, however, we are convinced and we have provided evidence that, despite some possible limitations, the FISH technique is a very good tool (possibly the best used so far) for improving the quantification of the living component of foraminifera and for better understanding their ecology in marine sediments.

The FISH (Fluorescent In Situ Hybridization) targets the rRNA inside living cells (and not the 18S rDNA gene). rRNA is the product of DNA transcription and a component of ribosomes, which are contained in living and actively metabolizing cells, as they perform protein synthesis. The FISH method involves the use of oligonucleotide probes which penetrate in cells previously permeabilized. The probes then enter the cell and specifically hybridize the target sequences of RNA present in the ribosomes. If there are no target sequences in the ribosomes, the probes do not hybridize the RNA and are subsequently eliminated by a subsequent washing step. Consequently, only specifically targeted cells will retain the probes under appropriate laboratory controlled, stringency conditions. Probes are labeled with fluorochrome (Cy3, fluorescein, etc, etc.) and the cells containing the hybridized probes can be easily observed under epifluorescence microscopy equipped with appropriate filters.

A higher number of ribosomes inside the cell (which is dependent upon the physiological status of cells) means a higher number of rRNA molecules. During FISH hybridization, each single probe will theoretically hybridize with a single molecule of rRNA. It is therefore expected that the larger the number of ribosomes in the cell, the stronger will be the fluorescence produced. As a result, the intensity of FISH signal is considered to represent an indication of cellular activity. Moreover, each cell will show a more o less homogenous fluorescence signal, depending of the concentration and localization of ribosomes inside the cells.

The use of the FISH approach for identifying active cells (which means those cells which have a high number of rRNA-containing ribosomes) is common since a decade in prokaryotic ecology (see Karner Fuhrman 1997 Appl Environ Microb and many other references) and has also applied to eukaryotic single-cell taxa (ciliates, such as Uronema sp., flagellates and mixed protists assemblages; Lim et al., 1993 and 1996). In theory, protists are potentially more appropriate for the use of oligonucleotide probes than Bacteria, because they contain a higher number of ribosomes (up to hundreds of thousands per eukaryotic cell versus 1.000 - 10.000 in a bacterial cell) and a higher concentrations of rRNA.

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We have implemented the amended version adding a new paragraph in which we have discussed in details limits and advantages of the FISH technique also in comparison with other methods currently utilized in foraminiferal studies, for discerning live/dead organisms (among which, Rose Bengal staining). We explained that the FISH technique can be used to identify the metabolically active fraction of benthic foraminiferal assemblages also in association with other methods (i.e. Cell Tracker Green), a method which conversely allows the identification of those cells able to enzymatically convert, inside the cell, non-fluorescent compounds into fluorescent ones.

As requested, we will also perform other FISH analyses to increase the number of assayed individuals, which will also include specimens from other taxonomic groups. These analyses will also include FISH assaying of cells which have exposed to different physiological stress, induced by changes in temperature and food supply. We will also explore in more detail the possibility to store the samples at -20°C for subsequent FISH analyses. We will collect additional sediment samples and to compare the results of FISH and CTG.

Finally, we will explain that one of the important advantages of the FISH method with respect to other "live/dead" methods: FISH can be applied both using a general (aspecific) probe able to hybridize to rRNA sequences of all foraminifera taxa, but can be designed to be more specific and target specifically a single species of foraminifera. In the future, the combination of this approach will have a huge potential as a tool to perform detailed quantitative and taxonomic studies on the abundance, biodiversity and distribution of the living fraction of specific foraminifera taxa. We believe this point represents one of the most promising aspects of this technique and will certainly have further developments in the near future.

We hope that these improvements, once incorporated in a new version of the manuscript, will make the ms suitable for publication on Biogeosciences.

LJ de Nooijer (Referee)

This manuscript published in BGD addresses an important and often underestimated problem in foraminiferal research. It is long recognized that distinguishing between living and dead foraminifera is very difficult and therefore, established methods may introduce biases in standing stocks, survival rates, etc. For this reason, a method that allows accurate discrimination between living and dead foraminifera is of vital importance to a wide variety of experiments and

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field studies.

The authors of the submitted manuscript have shown how an rRNA hybridization techÂňnique may be used to discriminate between living and dead foraminifers. The presented work, however, contains a number of serious issues that should be dealt with before the manuscript would be fit for publication. Implementation of all issues I found (listed below) may completely change the content and organization of the manuscript so that new reviews are likely to be necessary before acceptance in Biogeosciences.

Sincerely,

Lennart de Nooijer

Major concerns:

1-> I don't think that the optical test for determining whether foraminifera are alive or dead yields accurate results.

We partially agree with the Referee. Our aim was verifying, using the FISH technique, that organisms scored as "alive" under the optical microscope were truly "alive". rRNA (not rDNA) is a highly sensitive and labile molecule and this makes necessary to process the organisms shortly after sampling, to avoid changes in the physiological status of cells which may affect the rRNA content. It is clear that it is impossible to simultaneously test the same organism with other techniques (either RB, CTG, calcein or MTT) before performing the FISH assay. To discern live/dead foraminifera, we have used the only method (optical test) which was compatible with the further FISH testing of the same specimen.

We have tried to perform a comparison between FISH and Rose Bengal staining. However, FISH-stained Quinqueloculina and Ammonia individuals were not be stained by RB, suggesting that post-FISH staining of organisms with RB was not possible and could have led to unrealiable results.

2-> For species like Ammonia, we have often observed living cells that only filled up a few chambers (e.g. -5 out of -25: the other 20 were devoid of cytoplasm). Such living individuals display no pseudopodial activity unless they are kept for a number of days at high temperatures with abundant fresh food.

Such observations and previous reports suggest that (some) foraminifera may go into some sort

of inactive mode. Particularly with foraminifera collected in winter, this could introduce serious miscalculation of the living vs dead ratio of the population (fig 6). We will more clearly indicate how cultures were established in the paragraph 2.2 “Agnotobiotic cultures of foraminifera”. Our aim was to set up foraminiferal cultures and not a simply to maintain alive organisms present in the collected samples.

About the referee’s question on sediment samples, the sampled sites are monitored on a monthly basis by researchers at DiSMar (Dept. of Marine Science, Polytechnic University of Marche). In these surveys, faunal abundance (phytoplankton, foraminifera, meio- and macro-fauna) and main environmental parameters (T, S, Chl a, biochemical composition of organic matter) are investigated at each sampling time. Data about foraminifera abundance in February 2008 and 2009 (Bonatto et al., in prep) show that the benthic foraminiferal assemblages in the shallow Adriatic Sea displays a biomass of 122 and 129 μgC/10cm2 (volumetric method by Altenbach 1987 JFR 17: 106-109) modified by Kurbjeweit et al., 2000 (DSRII 47:2913-2955). These biomasses are higher than those of September 2008, December 2008 and January 2009, when values were comprised between 10 and 25 μgC/10cm2. Protein and biopolimeric C values, in February 2008 and 2009 (unpublished data) show a high (>1.5 mg g-1) content of bioavailable organic matter in sediments. Also, the high values of Chl-a content demonstrate fresh inputs of organic matter to the bottom. All these considerations let us hypothesizing that the benthic foraminiferal associations are alive and not under an “inactive mode” in the investigated area and period.

In addition, several papers have reported that the “optical” approach for distinguishing live/dead organisms is widely utilised: Filipsson et al. 2010 Biogeoscience 7:1335-1347; de Nooijer et al. 2008 Limnol. Oceanogr. Methods 6: 610-618 – “All specimens were screened for pseudopodial activity or presence of colored cytoplasm, indicating that individuals were alive at the start of incubation with HPTS”). de Nooijer et al. 2009 (Biogeosciences 6: 2669–2675, 2009) considered “alive individuals of Ammonia tepida if containing bright yellow protoplasm.” Bernhard et al. 2008 (DSRII, 55, 2617-2626) select living specimens on the basis of their appearance (so optical method) for adenosine triphosphate (ATP) analyses; empty tests ignored and specimens with apparent cytoplasm or sedimentary infilling considered.

Results from the ‘starvation experiment’ also show that the FISH-method may be biased by foraminifera that are in this possible ‘hibernation mode’.

BGD

7, C4211–C4220, 2010

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For the agglutinating species, visual clues may be even more problematic since, for instance, the color of their cytoplasm is easily distorted by the particles that make up their "shell".

For the tests conducted on agglutinated individuals, they have been picked up from foraminiferal samples maintenance settled in our laboratory (see paragraph 2.2). Their vitality has been tested under a microscope with a contrast-phase objective, which allows the observation of pseudopodial activity.

The authors should be aware of such errors and include comparison of their FISH method with an established one (i.e. CTG or BCECF-AM) to check if their results produce sensible living/dead ratios.

We will more clearly explain our findings. FISH and other staining methods cannot be performed on the same individual: the FISH protocol requires fixation and death of specimens, while the Cell tracker Green requires live and actively metabolizing cells. We have carried out comparisons between FISH and Rose Bengal staining. These will be included in the manuscript, including tables, numbers and data to better underline the FISH rationale (i.e. the identification of cells which are synthesizing proteins and are thus likely to be live and active) and its limitations. This will possibly include additional comparisons with RB staining method.

Alternatively, their presented method may be used to determine the metabolically active, rather than the living fraction of collected foraminifera. In that case, please omit discussion of living vs dead fractions.

We will change this section as suggested (please see above and the letter to the editor). Use of 'brightness' from pictures is problematic (section 3.4; fig 5). The fluorescent signal that the authors recorded depends on a number of things including the position of the focal plane, thickness of the shell wall, position of the foraminifer, presence of fluorescent stain in the surrounding medium, etc. Instead, pictures taken may be used to illustrate variability in fluorescence intensity or general differences between groups of foraminifers.

We will verify this suggestion about the position of the focal plane and thickness of the wall, in order to exclude the possible interference and/or signal amplification due to technical hitches.

However, we believe that a potential signal amplification cannot be affected by the position of the organism or the presence of fluorescent stain in the surrounding medium. Each micro-

BGD

7, C4211–C4220, 2010

scope slide has been prepared with one or few individuals, before the observation under the epifluorescence microscope. This allowed to avoid possible interferences between cells and the microscope. Moreover, FISH protocol include several washing steps to remove probe excess. We report frames by Bernhard et al., 1995 (J Euk Microbiol 42, 357-367) evidencing the advantage of epifluorescence microscopy in both transparent calcareous test (Ammonia) and transparent agglutinated species (Leptohalysis). Direct epifluorescence microscopy is recommended for identifying metabolically active specimens of tectinous foraminifera and species with transparent calcareous tests. This method can also be used for transparent agglutinated species (i.e. those with very thin walls or made up by mineral grains). If epifluorescence microscopy is used with opaque species (spectrofluorimetry is recommended for these types of foraminifera, see below), the specimen should be examined for cytoplasmic fluorescence near the aperture and as a thin veil of pseudopodia covering the exterior of the test. The possibility of a false-positive signal from bacterial contamination should always be considered, since bacteria are common epibionts of foraminifera [e.g. 8] and possess esterases [e.g. 51] that can cleave BCECF-AM or FDA [33, 39]. If spotty fluorescence is detected within the specimen, it could either be due to partitioned uptake of the probe into foraminiferal organelles (e.g. lysosomes) or to bacteria associated with dead cytoplasm. However, both the oligonucleotide probes utilized in our study are specific for eukaryotic organisms. Bacteria could not be detected with the used probes. Moreover, the differences in signal intensity could give important information regarding the metabolic state of cells (see section "Discussion"). Results, from FISH conducted on calcareous nannoplankton (Frada et al., 2006, J phycol, 42, 1162-1169) and on other organisms with a calcareous shell, show that probe fluorescence avoid the problems reminded above.

3-> The number of individuals for the 'washing' and 'dessication' protocols is too low to allow conclusions to be drawn: particularly with occasional hybridization (table 2). Right. We will add supplementary analyses to increase the significance of the results.

4 -> It is unclear what the difference is between the data presented in tables 1 and 3. Are these (partly) the same foraminifers? If there are two probes tested, which is used for the experiment presented in table 1? We apologize if the two Tables were not clear. In the amended version of the ms, they will be presented in a clearer way.

5 -> The discussion relies partly on results (lines 18-28, page 7487) that are not mentioned elsewhere in the manuscript.

BGD

7, C4211–C4220, 2010

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In the “Results” section, paragraph “Application of FISH to natural benthic foraminiferal assemblages”, we explained the Results obtained (lines 18-28, page 7487). We collected superficial sediments in two shallow water sites in the Adriatic Sea, in order to test the FISH protocol on natural assemblages to determine their living fraction. In the section “Discussion”, we then commented these results.

These results will be more clearly explained in the amended version, also on the light of the observed discrepancy between results obtained using FISH and Rose Bengal.

6 -> The main conclusions (i.e. that the presented method serves as a reliable way to discriminate between living and dead foraminifera; lines 24-29, page 7489) are not supported by the results.

This point is also discussed in the letter to the editor above.

We will add new analyses on cultures and natural sediment samples. This will be performed to confirm the usefulness of FISH as a tool to estimate the live fraction within benthic foraminiferal assemblages. Despite not being immune by limitations, we want to highlight that FISH can lead improvements in studies of foraminiferal ecology, including i) the evaluation of the metabolic state in cultured cells, ii) the analysis of natural benthic assemblages and iii) a higher reliability when compared with other assays, e.g. ATP analysis (Bernhard et al., 2008, DSRII, 55, 2617-2626).

We will add a paragraph discussing FISH limitations and its comparison with other methods available for determining live foraminifera. We will also discuss how FISH can be used in combination with Cell Tracker Green, which is based on a different rationale and is an indicator of viability, but is not devoid of limits and problems (it can lead to false-positive results, as enzymes responsible of the fluorescent signal can sometimes remain active even post-mortem).

Minor concerns:

1 -> Figure 1 can be deleted.

Ok, this will be done.

2 -> Describe in Methods what a ‘Autofluorescence assay’ is (table 1).

Please apologize for our lack of clearness. Before probe addition, we have verified the possible

BGD

7, C4211–C4220, 2010

autofluorescence emission by the tested organisms under the epifluorescence microscope and using the same light filter used for FISH. In that way, we wanted to exclude the possibility that some cellular components could emit autofluorescence, which would have lead to potential artifacts or misinterpretation of the FISH results.

3 -> How were the results of the two methods (optical and FISH) from table 1 in relation to each other? I.e. were all 21 'living' milliolids also identified as living by FISH? Or was there a larger mismatch between the two methods?

The results will be presented more clearly in the Tables.

4 -> Could the authors include more agglutinants in their analysis (table 1)?Would there be fluorescence from 'optically dead' specimens?

The results will be presented more clearly in the Tables.

5 -> I don't understand the added value of the SEM images (fig 2). Is it possible to test for organic remains by SEM?

SEM pictures have been used to help us in the interpretation of FISH results on washed shells. With the "Specimens washing" test, we wanted to test the possibility that the probe could hybridize also with the inner organic layer of the shell, the ectoplasm or other organic residues of recently-dead organisms. Non-specific staining of FISH probe can sometimes occur under non-stringent laboratory condition, thus potentially providing false-positive results for dead organisms (Hugenholtz, Tyson Blackall, in "Methods in Molecular Biology"). This test, along with the results from the application of the "nonsense probes", allowed us to demonstrate that non-specific incorporation of the probe was not occur in the foraminifera. The SEM allowed us to confirm that the complete organic lining(s) were dissolved within one hour using our protocols.

6 -> Why would the probe EUK1209R (line 2-3, page 7485) stain particles and organic material in agglutinating foraminifera?

We did not test agglutinated species with the EUK 1209R probe, in order to avoid the potential generation of false positive results. Agglutinated foraminifera construct their shells by attaching sediment particles which can, at least theoretically, contain some cytoplasmatic residues from other eukaryotic organisms living in the sediment. Despite rRNA is believed to be an extremely labile molecule when outside the cell, it is likely that organisms may contain rRNA from other

BGD

7, C4211–C4220, 2010

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organisms in their shell. As such, we suggested to perform FISH on agglutinated components of the foraminiferal assemblages using only foraminifer-specific “S17” probe, which guarantees that the fluorescence is truly emitted by the rRNA of the foraminifera.

7 -> Most of section 3.5 should be in the Methods and not in the Results.

Ok, we will change the ms accordingly.

8 ->The Discussion contains large parts that belong to the Introduction. For example, disadvantages of previously developed methods is the rationale for the experiments presented by Borrelli et al. and need not to be repeated in the Discussion. Instead, the Discussion should address uncertainties in the proposed method and e.g. the applicability to (large) volumes of sediment.

Ok, we will change the ms accordingly.

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