Biogeosciences Discuss., 7, 7475–7503, 2010
www.biogeosciences-discuss.net/7/7475/2010/
doi:10.5194/bgd-7-7475-2010


Biogeosciences Discussions



# Determination of the metabolically active fraction of benthic foraminifera by means of Fluorescent in situ Hybridization (FISH)

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Received: 1 October 2010 – Accepted: 4 October 2010 – Published: 13 October 2010

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Published by Copernicus Publications on behalf of the European Geosciences Union.







## Abstract

Benthic foraminifera are an important component of the marine living biota, but protocols for investigating their viability and metabolism are still extremely limited. Classical studies on benthic foraminifera have been based on direct counting under light microscopy. Typically these organisms are stained with Rose Bengal, which binds proteins and other macromolecules, but this approach does not allow discriminating between viable and recently dead organisms. The fluorescent in situ hybridization technique (FISH) represents a potentially useful approach identifying living cells with active metabolism cells. In this work, we tested for the first time the suitability of the FISH technique based on fluorescent probes targeting the 18S rRNA, to detect these live benthic protists. The protocol was applied on the genus *Ammonia*, on the Miliolidae group and an attempt was made also with agglutinated species (i.e., *Leptohalysis scottii* and *Eggerella scabra*). In addition microscopic analysis of the cytoplasm colour, presence of pigments and, sometimes, those of pseudopodial activity where conducted. The results of the present study indicate that FISH targeted only live and metabolically active foraminifera. These results allowed to identify as "live", cells improperly classified as "dead" by means of the classical technique (Type I error) and vice versa to identify as dead the foraminifera without rRNA, but stained using Rose Bengal (Type II error). In addition, the comparative FISH analysis of starved and actively growing cells demonstrated that individuals with active metabolism were stained more intensively than starved cells. This finding supports the hypothesis that the physiological status of cells can be directly related with the intensity of the fluorescent signal emitted by the fluorescent probe. We conclude that the use of molecular approaches could represent a key tool for acquiring crucial information on living foraminifera specimens and for investigating their ecological role in marine sediments.





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## 1 Introduction

Foraminifera, a group of testate protists, which in deep-sea ecosystems can become a dominant component of the benthic fauna both in terms of abundance and biomass (Gooday et al., 1998). Since hard-shelled foraminifera are largely used for paleoenvironmental reconstructions, the knowledge of the fossil assemblages is quite advanced (e.g. Schmiedl and Mackensen, 1997; Katz et al., 2003; Morigi et al., 2005; Morigi, 2009; Murray, 2006), but current biological and ecological studies are still very limited due to the lack of reliable protocols for studying their metabolism and for distinguishing living from dead cells within the assemblages. Over the last 20 years, new methods have therefore been developed to distinguish between living and dead foraminifera, with different degrees of accuracy (Bernhard, 2000). Rose Bengal, a non-vital staining, has been widely used in ecological studies to recognize presumably dead (unstained) foraminifera from the living (stained) counterparts (Murray and Bowser, 2000). However, it is known that within dead animals parts of their tissues may remain preserved even for a long time (Murray and Bowser, 2000) and since the Rose Bengal stain binds proteins and other macromolecules the potential of false positive cells is high. Other approaches to detect metabolically active foraminifera are based on fluorogenic probes (e.g., diacetates of fluorescein – FDA- and AM-esters of biscarboxyethylcarboxyfluorescein – BCECF-AM-; Bernhard et al., 1995), that measure both enzymatic activity, which is required to activate their fluorescence, and cell-membrane integrity. However, enzymatic activities might be present after the death of the organisms and their use in shelled organisms appear to be potentially problematic. Also the use of the enzymatic reduction of the salt MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide or thiazolyl blue, which produces a reddish purple crystal) has been recently proposed as a viability assay for living foraminiferal species from shallow water systems (De Nooijer et al., 2006). But this approach produces false positives and the need of perform short-term laboratory experiments. Other probes, such as CellTracker Green CMFDA (CTG), allow detecting actively metabolizing organisms by identifying





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the presence of respiratory activity, either in the laboratory or in situ (Bernhard et al., 2004, 2006; Pucci et al., 2009) and can be applied to both unicellular and multicellular marine organisms (Danovaro et al., 2010). However, the use of CTC is time-consuming (because of the need of incubating samples up to 19 h with the fluorescent molecule), requires live-dead controls experiments and the long incubation may alter the physiological conditions or even affect the survival of the incubated foraminifera. In addition, the CTG fluorescence can be masked by the autofluorescence of the foraminiferal shell or other sources of fluorescence (Pucci et al., 2009).

The Fluorescent in situ Hybridization (FISH) is a novel molecular technique based on the use of fluorescently-labelled, rRNA-targeted oligonucleotide probes, which hybridize target molecules and are visualized under epifluorescence microscopy (Dupperon et al., 2005). The use of FISH for identifying the single cells has been widely utilised in prokaryotes for a variety of habitats, including marine sediments and soils (Karner and Fuhrman, 1997; Christensen et al., 1999). Despite its large use in the study prokaryotes, targeting the 16S rRNA (Hugenholtz et al., 2001; Danovaro et al., 2009), FISH based on 18S rRNA as a target molecule has been also applied to investigate marine picoeukaryotes (Not et al., 2002), marine protists (including ciliates such as *Uronema* sp., flagellates including *Cafeteria* sp. and mixed protists assemblages; Lim et al., 1993, 1996) and the eukaryotic symbionts of some marine invertebrates (e.g., *Symbiodinium* spp.; Yokouchi et al., 2003). A development of FISH targeting marine eukaryotes is represented by the COD-FISH, which enable to identify calcifying microorganisms (Frada et al., 2006). Morever, the association of SEM and FISH analyses largely improves the potential of taxonomic and morphological characterization of a wide range of protists (e.g., alveolates, stramenopiles, kinetoplastids and cryptomonads; Stoeck et al., 2003). Despite the wide potential application of this technique, no FISH protocols have never been set up and tested to distinguish living organisms.

The aim of this work was to test the suitability of FISH for the quantification of living foraminifera in marine sediments in a more efficient, simple and rapid way than other available methods utilised so far.





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## 2 Materials and methods

### 2.1 Study site and sediment sampling

Sediments were collected from two shallow water sites (Falconara and Portonovo) located in the Central Adriatic Sea (Fig. 1), in October 2009 and in February 2010, using a Van Veen grab. Only the top 2 cm of the sediment, containing the highest abundance of living foraminifera, was collected. The sediment was stored into a plastic box filled with seawater (collected from the same site) and carried to the laboratory within 2–4 h, where it was immediately processed. The Falconara site is characterized by the following environmental and biochemical conditions: $1.52 \pm 0.20\,\mu g\,g^{-1}$ of chlorophyll-*a*, $9.81 \pm 0.79\,\mu g\,g^{-1}$ of phaeopigments, $0.87 \pm 0.06\,mg\,g^{-1}$ of proteins and $0.65 \pm 0.05\,mg\,g^{-1}$ of biopolymeric carbon in the sediment (biochemical analyses performed as described by Pusceddu et al., 2009). The Portonovo site is characterized by a chlorophyll-a concentration of $2.57 \pm 0.47\,\mu g\,g^{-1}$, $16.08 \pm 2.8179\,\mu g\,g^{-1}$ of phaeopigments, $0.68 \pm 0.06\,mg\,g^{-1}$ of proteins and $0.66 \pm 0.01\,mg\,g^{-1}$ of biopolymeric carbon.

### 2.2 Agnotobiotic cultures of foraminifera

Sediments were stored at in situ temperature (19 °C in October, 9 °C in February) and salinity (36‰, constant during the sampling) and immediately transferred to the laboratory to reduce possible stress to the organisms. Two culture sets were prepared on airtight boxes. During the growth, chemical and physical parameters were maintained at a pH of 8, temperature of 23 °C and salinity of 36‰. To avoid evaporation and to maintain stable salinity within the cultures, small quantities of filtered (0.45 µm) seawater, properly mixed with distilled water, were added every week. Cultures were fed, every two weeks, with a mixture of algae (*Dunaliella parva, Chlorella* sp. and *Isochrysis* sp., Heinz, 2001; Heinz et al., 2001). Prior to injection in the cultures, the algae were treated in an ultrasound bath. A set of starved cultures (i.e., not fed with any type of algae) was also prepared for some experiments. In this case, only specimens belonging





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to Miliolidae group were cultivated.

### 2.3 Analysis of the natural benthic foraminiferal assemblages

Sediments collected from the two sites in February 2010 were analysed for the determination of total foraminiferal abundance versus the abundance of the living fraction (determined either by assessing pseudopodial development and the FISH method). In this experiment, we counted all the foraminiferal association (dead plus alive). Then we analyzed the living fraction under an optical microscope and with FISH protocol. For this comparison, we considered only foraminiferal specimens that showed pseudopodial activity and presence of algal pigments.

### 2.4 Development of the FISH protocol

For in situ hybridization on foraminifera, we adapted to the protocol routinely used for marine prokaryotes and other microbial eukaryotes, such as ciliates and dinoflagellates (Karner and Fuhrman, 1997; Hugenholtz et al., 2001; Not et al., 2002; Stoeck et al., 2003; Yokouchi et al., 2003; Mikulski et al., 2005; Frada et al., 2006). Briefly, all foraminifera were picked up from the cultures, prepared in our laboratory, and/or from sediment samples. Specimens were considered as alive on the basis of cytoplasm colour (from yellow to light brown), presence of pigments and/or the presence of detectable pseudopodial activity under an optical microscope provided with a phase contrast objective. Otherwise cells that did not show any of these features were considered as dead. To test the efficiency of FISH over different foraminiferal taxonomic groups, we selected specimens of *Ammonia* and of the Miliolidae group. Analyses were also performed on agglutinated species, such as *Leptohalysis scottii* and *Eggerella scabra*. To optimize the procedure, we compared two oligonucleotide probes: Euk1209R (5′-GGGCATCACAGACCTG-3′), which is a widely used universal eukaryotic probe already used for FISH analysis (Lim et al., 1993; Not et al., 2002; Stoeck et al., 2003) and S17 (5′-CGGTCACGTTCGTTGC-3′), which is a probe





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specifically designed for foraminifera and previously used for phylogenetic analyses on this group (Pawlowski, 2000; Holzmann et al., 2001; Pawlowski and Holzmann, 2002; Garcia-Cuetos et al., 2005). According to Pernthaler et al. (2001) and Hugenholtz et al. (2001), non-sense probes "non-Euk1209R" and "non-S17" (i.e. the reverse complement of probes Euk1209R and S17, respectively) were used as negative controls. These probes have no known rRNA target and are used to check for the non-specific incorporation of the probe into the cells. Prior of the development of the FISH protocol, foraminiferal individuals have been examined for autofluorescence (controls without probe addition) in order to exclude that cellular components (e.g., photosynthetic pigments, cofactor F420 and some proteins) displayed autofluorescence, which can lead to artifacts in the analysis of the FISH fluorescence (Hugenholtz et al., 2001).

For FISH analysis, the same individuals previously identified as living or dead using optical microscopy were fixed and analysed as described here below. One to five specimens of foraminifera were placed in a concave slide for taxonomical identification. Water was removed from the slide and the specimens fixed with 50 µl of phosphate-buffered saline (PBS) and ethanol (1:1 vol/vol). No formaldehyde was used do to the potentially negative effects on the hybridization procedure. Fixed samples were immediately analysed or, when this was impossible, stored at −20 °C for up to one week. For hybridization protocol, the PBS:ethanol mixture was removed by exposure to LAF (Laminar Air Flow) for a few minutes to dry. Then, 40 µl of a mixture (9:1 vol/vol) of hybridization buffer [5M NaCl, 1M Tris/HCl, 35% formamide and 0.01% SDS] and probe solution (0.5 ng µl$^{-1}$ working concentration) was added. This working concentration is 100-times lower than the one suggested in literature (50 ng µl$^{-1}$; Hicks et al., 1992). The reason for this choice was that the use of a concentration of 50 ng µl$^{-1}$ often resulted in the presence of fluorescence also in the dead organisms (data not shown). The percentage of formamide was chosen based on literature data (for the Euk1209R probe; Stoeck et al., 2003) and on the results of preliminary experiments for the definition of the optimal stringency (for the probe S17; data not shown). Probes (including non-sense probes) had been previously labelled at 5' end with the fluorescent molecule





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Cy-3 (Eurofins MWG Operon). After hybridization, slides were placed into a Petri dish and put in a dark incubation chamber. Within the Petri dishes, we also placed a small piece of a paper towel soaked with 1 ml of hybridization buffer, to prevent buffer evaporation from the samples. Hybridization times varied from 1.5 to 3 h at 46 °C. After incubation, the hybridization mixture was removed and 200 µl of pre-warmed (at 46 °C) washing buffer [5M NaCl, 1M Tris/HCl, 0,5M EDTA, 0.01% SDS] were added. The slides were incubated at 48 °C for 15 min and then the solution carefully removed using a pipette. The slides were rinsed with sterile milliQ water and then dried under LAF. The slides were then observed under an epifluorescence microscope (Zeiss Axioskop 2, magnification ×100 using a dry objective) using an appropriate filter set for Cy-3.

### 2.5 Specimens washing

In order to test the possibility that the probe could hybridize also with the inner organic layer of the shell, the ectoplasm and other organic residues of recently-dead organisms (Weiner and Erez, 1984; Bentov and Erez, 2006), were treated with different washing procedures to remove the organic matrix. Two different protocols were tested: (i) drying shells at 60 °C for one hour ("desiccation protocol"); and (ii) one hour incubation in 3% NaClO and subsequent washing (7 steps) in deionised water ("washing protocol"). The desiccated and washed shells were then analysed using the FISH protocol described above. The washed shells were also observed under a SEM microscope for testing the effectiveness of the washing protocol (Fig. 2a, b, e and f).

### 2.6 Visual image analyses

To compare differences in fluorescence emission between cells under different physiological and metabolic regimes (e.g., fed versus starved cells), digital images of hybridized foraminifera were acquired, with fixed settings and no scaling, using a Lumix TZ-65 10.1 megapixels digital camera (Panasonic) attached to the epifluorescence





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microscopy. This approach is similar to that already used for measuring the uptake rates of fluorescent bacteria by foraminifera (Langezaal et al., 2005). The amount of brightness in each picture was taken as a measure of fluorescence emission following hybridization. Brightness was measured using Adobe PhotoShop CS2 and the option "Color Picker". For each analysed specimen, twenty different measurements were taken in randomly-chosen areas of the complete individual and the average values plus standard deviations was calculated.

## 3 Results

### 3.1 Autofluorescence

The results of FISH analyses performed on the different taxonomic groups, including the autofluorescence analysis of foraminifera, are summarized in Table 1.

### 3.2 FISH on washed shells

Both cultured and wild dead foraminifera belonging to *Ammonia* genus and Miliolidae group were observed under SEM after treatment for removal of the organic remains (desiccated and/or washed using the protocols described above). Our results showed that the "washing protocol" was highly efficient in removing all cytoplasmatic remains in both dead *Ammonia* (Fig. 2a–b; Table 2) and Miliolidae (Fig. 2e–f; Table 2). In fact, in the "washing" protocol we hybridized 12 organisms, 5 specimens were hybridised with the Euk1209R and 7 specimens with the S17 probes; see Table 2 for taxonomic groups). Emission of fluorescence was again null or, when detectable, extremely low and therefore indistinguishable from the background fluorescence (Fig. 2d for Ammonia and 2g–h for Miliolidae). Only the "desiccation" protocol produced 5 cases of false positive for the Miliolidae group, hybridized with S17 specific probe. For this reason this treatment was considered unsuitable for further analyses.





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In the "washing" protocol we observed only one case of organism, belonging to Miliolidae group, when hybridized with the S17 probe, displayed a fluorescent signal (Table 2). Due to this result, the procedure was modified by increasing the incubation time in 3% NaClO from 1 to 2 h. This procedure allowed to eliminate any fluorescent signal in all of the specimens of Miliolidae group subsequently analyzed. These steps allowed to set up a FISH protocol unbiased by false positives.

### 3.3 FISH on living and dead cells

The FISH protocol was applied using the non-sense probes (nonEuk1209 and nonS17) to both living and dead organisms collected from all the investigated coastal sediments. None of the investigated foraminifera was stained (Fig. 3), indicating that non-specific probe binding did not occur in the in situ hybridization analysis (Table 3). Living and dead foraminifera belonging to the *Ammonia* genus and Miliolidae group, as well as to the specie *Eggerella scabra* and *Leptohalysis scottii*, were firstly identified as living or dead (based on conventional microscopic analyses) and subsequently treated for FISH analysis. Examples of typical hybridization results are shown in Fig. 4a–f. Overall 69 individuals were analyzed. Of the 19 individuals belonging to the *Ammonia* group (EUK1209R probe), 17 emitted fluorescence. Of 7 individuals belonging to the *Ammonia* group treated with the S17 probe, 4 emitted fluorescence and were classified as living cells (Table 3). Overall, 2 cells hybridized with the EUK1209R probe, and 3 cells hybridized with the S17 probe, did not emit fluorescence. The test made with 3 organisms of the *Ammonia* group with the EUK1209R (1 cell) and S17 (2 cells) non-sense probes revealed the lack of any fluorescence.

35 foraminifera, belonging to Miliolidae, were also investigated: 22 out of the 25 hybridized with EUK1209R and 4 out of 6 hybridized with S17 probes were identified as living. Also in this case some cells did not emit fluorescence (i.e., 3 cells hybridized with EUK1209R and 2 hybridized with S17, Table 3). Finally, 2 specimens of the Miliolidae group were hybridized with the EUK1209R and 2 with S17 non-sense probes providing, in both cases, any fluorescence emission.





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We tested the FISH protocol also on 2 agglutinated species: *Eggerella scabra* ($n = 3$) and *Leptohalysis scottii* ($n = 2$) using only the probe S17. The universal EUK1209R probe was not used to avoid possible staining of agglutinated particles shell and organic material. One specimen of *Eggerella,* classified as living using by optical microscopy analysis did not emit fluorescence. All cells of *Leptohalysis*, classified as living by optical microscopy were confirmed by FISH analysis (Table 3). All living individuals hybridized showed an intense fluorescence emission (Fig. 4b, d and f), while dead individual provided null or very low background fluorescence.

In both *Ammonia* and Miliolidae individuals, the fluorescence was apparently homogeneous in the cell protoplasm also if, in some cases, in the genus *Ammonia* the fluorescence was concentrated in the last chamber or in the first chambers and in Miliolidae, the fluorescence was highest along the edge of the shell. In individuals belonging to *Eggerella scabra* and *Leptohalysis scottii*, the fluorescence appeared more or less homogeneously distributed within the organisms, and with a signal intensity higher than in *Ammonia* and Miliolidae.

Different FISH probes were utilized (the universal Euk1209 and the more specific S17), which showed a comparable efficiency in the staining of living individuals. However, our results indicate that cells stained with the S17 probe generally emitted a more intense fluorescence than those hybridized with the universal eukaryotic probe (EUK1209). This evidence was also confirmed by visual image analyses (Fig. 5).

### 3.4 Experiment on cultured cells

As the rRNA cellular content is known to be proportional to the growth rate of the cell, we expected that the physiological condition of foraminiferal cells would affect the amount of probe bound and consequently the intensity of the hybridization signal. To test this hypothesis, the FISH protocol was applied to two controlled foraminiferal cultures (see Materials and methods for details), with the aim of comparing the fluorescence emission between fed (2 cells) and starved (1 cell) organisms. In this case, cells were hybridized using the Euk1209R probe. The results clearly indicated that





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fluorescent intensity due to probe binding was much higher in fed than in starved organisms (Fig. 5). These comparative analyses revealed, on average, about 41% higher fluorescence intensity in fed than in starved specimens, indicating the fluorescent signal is dependent upon the physiological condition of the assayed cells.

### 3.5 Application of FISH to natural benthic foraminiferal assemblages

As a final step, we tested the ability of the FISH protocol to quantify the living fraction in foraminiferal benthic assemblages. Sediments were collected in the two study sites (Falconara and Portonovo). Also in this case, only the superficial (0–2 cm) layer was sampled. The sediment was stored into a plastic box filled with seawater (collected from the same site) and carried to the laboratory within 2–4 h, where it was immediately processed. A total abundance of 62 and 158 individuals were collected from sediment samples in Falconara and Portonovo, respectively. The analysis of these two assemblages revealed that 9 and 3% (from Falconara and Portonovo, respectively) was defined as living based on presence of pseudopodial activity, pigments inside the cell and cytoplasm colour (Fig. 6a) whilst, according to the FISH protocol, 6 and 3% of the individuals were identified as alive (Fig. 6b).

## 4 Discussion

In the last years, one of the most controversial issues in the analysis of benthic foraminifera has been distinguishing living from dead foraminifera (Bernhard, 2000). A variety of different methods has been proposed. These methods can be divided into terminal approaches, causing the death of the organisms investigated and non-terminal approaches, which leave the organism alive (Murray and Bowser, 2000). However, all the available approaches suffer of conceptual and/or practical weaknesses, which do not provide certainty of identification and/or hamper the application of these protocols in routine analysis. As a result, we still miss a reliable protocol for the identification of





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living foraminifera in natural sediments.

The aim of this work was to develop of a new terminal approach based on the development of a molecular protocol, FISH, which allows a rapid discrimination between dead and living foraminifera. This approach has been extensively utilised for the study and identification of the living fraction of marine prokaryotes and applied also to different unicellular eukaryotes (Lim et al., 1993; Hugenholtz et al., 2001; Not et al., 2002; Stoeck et al., 2003; Frada et al., 2006), but has been never utilised in foraminifera.

Before starting with experiments, autofluorescence were tested. Autofluorescence is known for a wide range of prokaryotic and eukaryotic organisms, including nematodes, phytoplankton cells and stony corals (Aubin, 1979; Booth, 1987; Forge and MacGuidwin, 1989; Ainsworth et al., 2006). Autofluorescence may confound FISH analysis, as it might make uncertain the actual source of fluorescence. Our results revealed that all specimens of all genera/species investigated (genus *Ammonia*, the Miliolidae group, and the *Eggerella scabra* and *Leptohalysis scottii* species) showed null or extremely weak autofluorescence under the filter set used for Cy-3 detection, suggesting that autofluorescence is not a problem in the analysis of foraminifera using the FISH protocol.

The results obtained by the use of the FISH are different than those obtained using the two methods traditionally utilised for discriminating the living fraction (i.e., the intracellular analysis by light microscopy and the pink coloration obtained using the Rose Bengal staining). When compared with the analysis of the cytoplasm colour and presence of pigments by light microscopy (without investigating pseudopodial activity), FISH classified in the 16% of the case as “living”, cells classified as “dead” by light microscopy. Conversely, when FISH analysis was compared with Rose Bengal staining, a large fraction of cells colored by Rose Bengal were devoid of rRNA as did not respond to the hybridization. Since the controls made allowed to test that the FISH was effective on living specimens these results indicate that Rose Bengal provide an overestimations of the living fraction of foraminifera in marine sediments. The results of the FISH approach represented suggest that this is a reliable tool for determination





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of living foraminifera in marine sediments. Among the potential alternatives, the analysis of the ATP content (DeLaca, 1986), is not only time consuming but, most importantly, highly imprecise, as the amount of ATP can vary significantly upon the taxon, the size and the metabolic status and activity of the individual investigated (Linke et al., 1995). FISH is clearly more effective than Rose Bengal staining, which is known to stain also dead foraminifera even after months or years after their death (Bernhard et al., 2006). In fact, once the organism die, his cellular rRNA is subjected to almost immediate degradation, so that this approach can provide real time information of the abundance of living and active cells. The FISH method can also be advantageous to the recently-developed method based on thiazolyl blue (MTT) reduction (De Nooijer et al., 2006) which, besides the advantages of reacting to the enzymatic activity of the living cells, can be seriously biased by the presence of living prokaryotes within the dead foraminifera, as prokaryotes can reduce MTT as foraminifera do and lead to false positives. For this reason authors need incubating the samples with antibiotics (to inhibit bacterial growth). This bias cannot occur using the eukaryotic probes utilised in the present study. In addition, the FISH protocol has two main advantages when compared with fluorogenic assays based on FDA and BCECF (Bernhard et al., 1995) or the vital fluorogenic stain CellTracker Green (Bernhard et al., 2006, 2009; Pucci et al., 2009) as the protocol described here is much less time consuming (does not need incubation of living foraminifera) and allows to store the samples immediately after collection at −20 °C (thus permitting the collection and analysis of a much larger number of samples).

The cellular rRNA content is known to be related to the growth rate, so that the fluorescence intensity from ribosomal RNA probe binding may also serve as a relative measure of activity, growth of the cell and/or of protein synthesis (DeLong et al., 1989). A low FISH signal intensity has been proposed to correspond to dormant cells or low metabolism in marine prokaryotes (Karner and Fuhrman, 1997). In our study, the comparison between fed and starved organisms in cultures confirmed that the fluorescence intensity of living foraminifera is positively correlated with their physiological conditions





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as a higher intensity is associated to a higher rRNA content in fed organisms versus a lower amounts of rRNA in starved cells (Fig. 5).

Differences were also observed in the fluorescence intensity when living specimens belonging to different taxa were investigated. Fluorescence intensity was generally more intense in *Eggerella scabra* and *Leptohalysis scottii* than in *Ammonia* and Miliolidae. These differences in fluorescence emission can be possibly explained by differences in the shell structure and composition. In fact, *Eggerella* and *Leptohalysis* display an agglutinated wall structure, which is composed by sedimentary particles cemented by organic molecules (Boersma, 1998), while *Ammonia* and Miliolidae are characterised by a thick, calcareous test. In particular, *Ammonia* presents a low-Mg content, bilamellar and perforate calcite test (Sen Gupta, 1999), whereas the Miliolidae have generally a non-lamellar, non-perforated porcellaneous test, composed by rods/laths of calcite with high-Mg content. The calcite units of the wall are randomly arranged within the embedding organic material (Hansen, 1999). The low fluorescence intensity of the Miliolidae could be then related to different shell composition and organization (with a highly compact wall structure) and or to the different probe penetration due to the absence of the pores in their shells (Fig. 4).

Finally, the FISH protocol was used to gather ecological information on the living fraction within benthic foraminiferal assemblages in marine sediments. The results of our study indicate that, according to the FISH protocol, only a minor fraction of the assemblages (3–6%) was alive. This result indicates that FISH protocol can be applied to field studies as it gives results consistent with those obtained from direct microscopic observation of the living specimens.

The results of all our investigations show that the FISH protocol here developed is highly efficient and allows to reliably discriminate between living and dead cells in both cultures and natural marine sediments. The future combination of this method with group-specific probes has a huge potential as a tool to perform detailed quantitative and taxonomic studies on the abundance, biodiversity and distribution of the living foraminifera.





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*Acknowledgements.* This work was partially supported by HERMES - Hotspot Ecosystem Research on the Margins of European Seas (contract number GOCE-CT-2005-511234), funded by the European Commission's Framework Six Programme and HERMIONE – Hotspot Ecosystem Research and Man's Impact on European Seas (contract number 226354) funded by the European Commission's Framework Seven Programme. The authors gratefully acknowledge Antonio Pusceddu, Silvia Bianchelli and Antonio Dell'Anno (Polytechnic University of Marche) for support in the biochemical analyses and precious advices on an early draft of the manuscript and Annachiara Bartolini for the photos at SEM. The first author gratefully thanks also the Cushman Foundation for the Cushman Foundation Student Travel Award for Forams 2010.

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**Table 1.** Results of FISH analyses performed on the different taxonomic groups. (*) Autofluorescence analysis was performed on the specimens belonging to the four taxonomical groups analysed before FISH assay.

| | Numbers of individuals | Autofluorescence assay (*) | Alive/dead (Optical Microscopy) | Alive/dead (FISH) |
|---|---|---|---|---|
| *Ammonia* genus | 26 | NO | 15/11 | 21/5 |
| Miliolidae | 31 | NO | 21/10 | 26/5 |
| *Eggerella scabra* | 3 | NO | 3/0 | 2/1 |
| *Leptohalysis scottii* | 2 | NO | 2/0 | 2/0 |
| Total organisms | 62 | | 41/21 | 51/11 |





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**Table 2.** Results of FISH analyses performed on the shells after treatment with "desiccation" and "washing" protocol and hybridization with EUK1209R and S17 probes.

| EUK 1209R probe | Num. cells | FISH result (YES/NO) |
|---|---|---|
| "Washing" protocol *Ammonia* group | 3 | NO |
| "Dessication" protocol *Ammonia* group | 1 | NO |
| "Washing" protocol Miliolidae | 2 | NO |
| "Dessication" protocol Miliolidae | 3 | NO |
| S 17 probe | | |
| "Washing" protocol *Ammonia* group | 4 | NO |
| "Dessication" protocol *Ammonia* group | 1 | NO |
| "Washing" protocol Miliolidae | 3 | 1 YES–2 NO |
| "Dessication" protocol Miliolidae | 5 | YES |





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**Table 3.** Results of FISH analyses performed on the different taxonomic groups. Comparison between the EUK1209R and S17 probes results. (*) Organisms tested with EUK1209R and S17 non-sense probe as negative control. See text for details.

| | Number of individuals | Alive EUK1209 probe | Dead EUK1209 probe | Alive S17 probe | Dead S17 probe | Non-sense probes (*) |
|---|---|---|---|---|---|---|
| *Ammonia* group | 29 | 17 | 2 | 4 | 3 | 3 |
| Miliolidae | 35 | 22 | 3 | 4 | 2 | 4 |
| *Eggerella scabra* | 3 | None | None | 2 | 1 | None |
| *Leptohalysis scottii* | 2 | None | None | 2 | 0 | None |
| Total organisms | 69 | | | | | |





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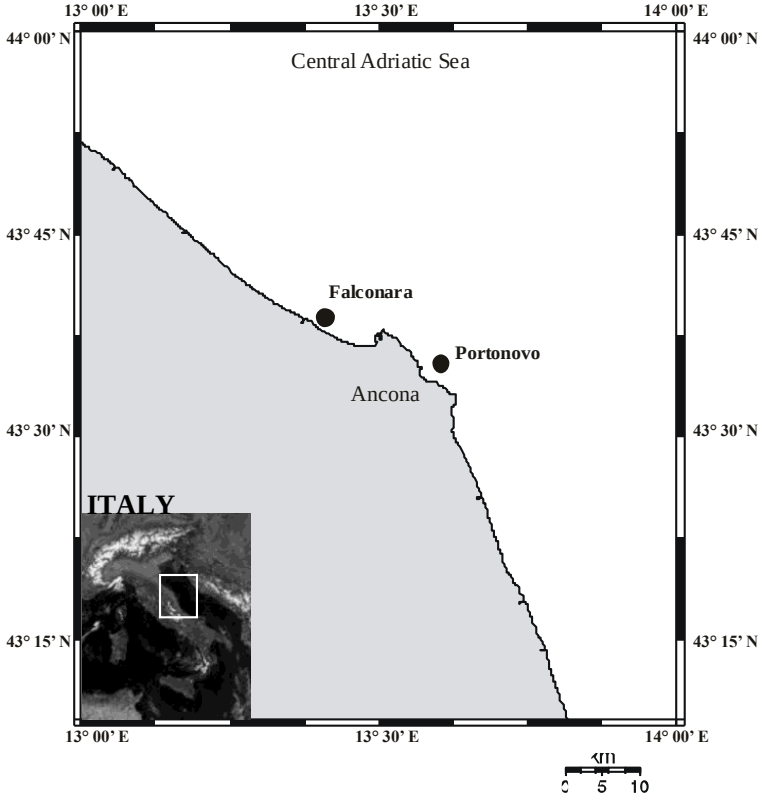


**Fig. 1.** Map showing the location of sampling sites.





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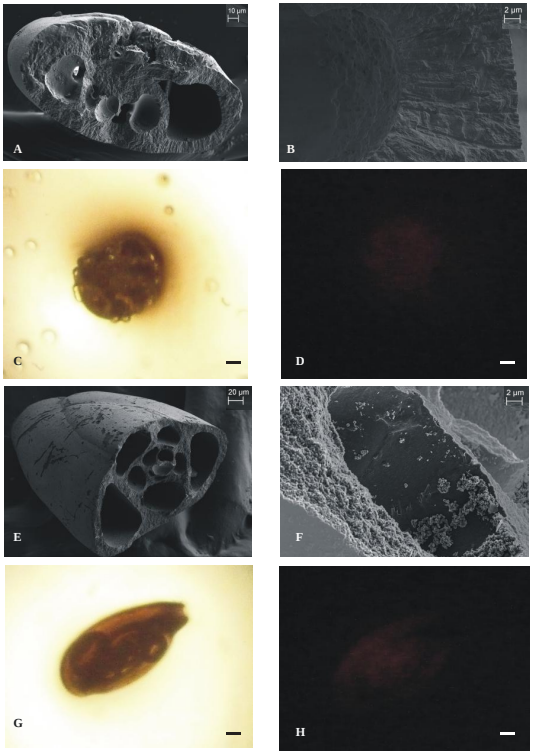


**Fig. 2.** Specimens treated with "washing protocol" and, after that, analyzed with FISH. No fluorescence occurs. See text for details. **(A–D)** *Ammonia* sp. analyzed by SEM, after the "washing protocol" (A, shell section; B, particular); optical (C) and epifluorescence microscopy after hybridization with the S17 probe (D); **(E–H)** Miliolidae group individual analyzed by SEM, after the "washing protocol" (E, shell section; F, particular), optical (G) and epifluorescence microscopy after hybridization with the S17 probe (H). Scale bars = 12.5 µm except where stated.





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Metabolically active fraction of benthic foraminifera by means of FISH

C. Borrelli et al.



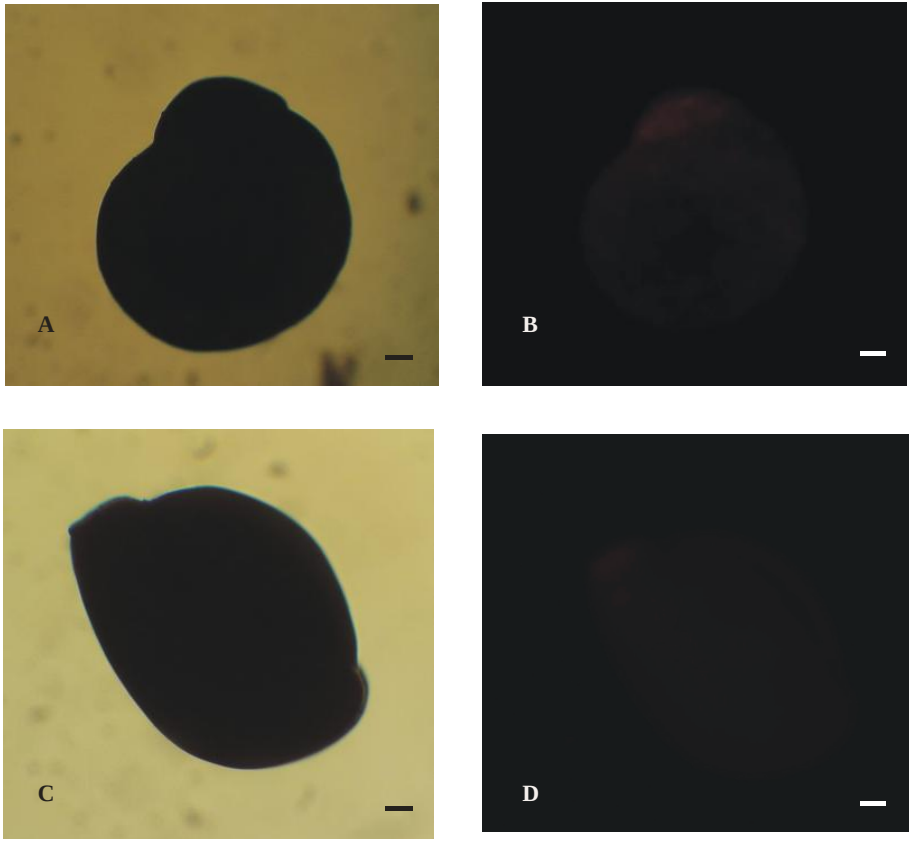


**Fig. 3.** Specimens analyzed after hybridization with non sense S17 probe. No fluorescence occurs. See text for details. **(A–B)** *Ammonia* sp. analyzed by optical (A) and epifluorescence (B) microscopy; **(C–D)** Miliolidae group individual viewed under optical (C) and epifluorescence (D) microscopy. Scale bars = 12.5 µm.





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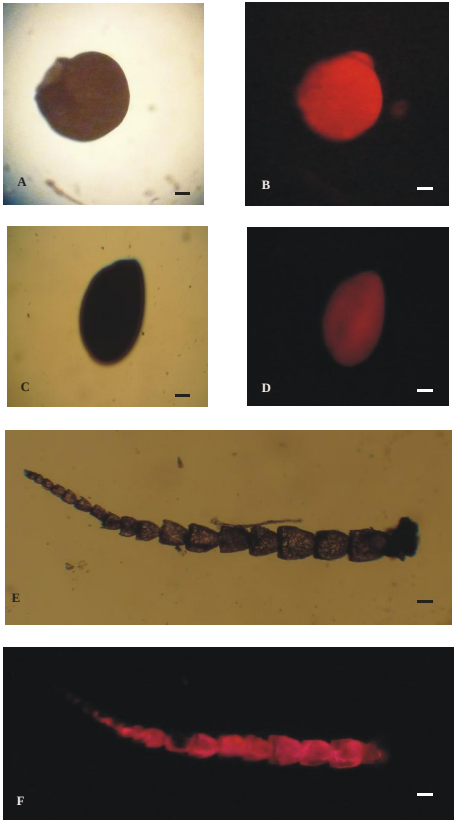


**Fig. 4.** **(A–B)** *Ammonia* sp. analyzed by optical (A) and epifluorescence microscopy after hybridization (B); **(C–D)** Miliolidae group individual under optical (C) and epifluorescence microscopy after hybridization (D); **(E–F)** *Leptohalysis scottii* under optical (E) and epifluorescence microscopy after hybridization (F). Scale bars = 12.5 µm. For FISH analyses, the S17 probe was used.





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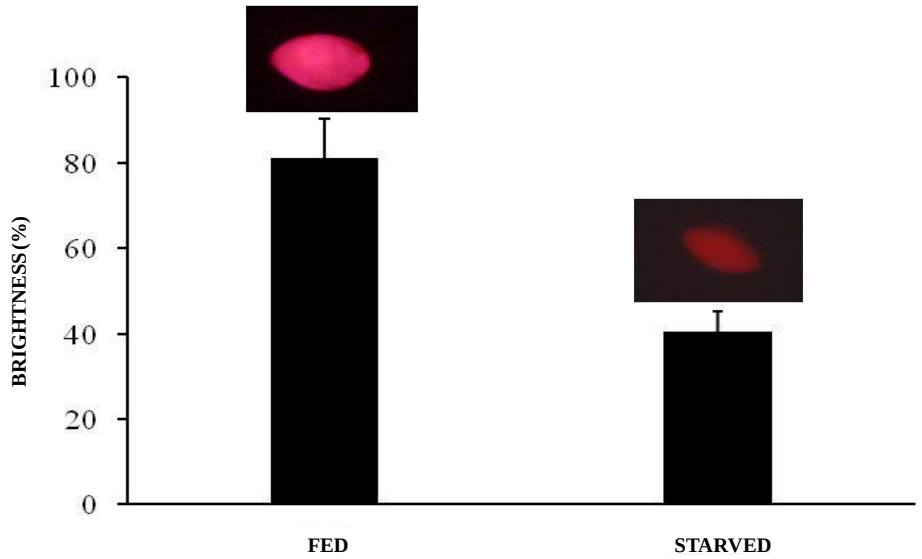


**Fig. 5.** Difference in fluorescence emission between fed and starved *foraminifera* (as determined from visual image analyses). Standard deviation is reported.





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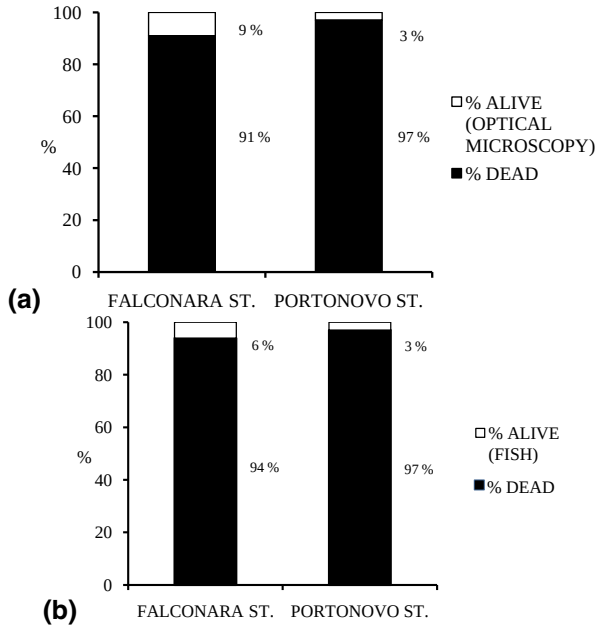


**Fig. 6.** Total abundance of benthic foraminifera in Falconara and Portonovo samples. Living fraction determined by optical microscopy **(a)** and FISH **(b)**.





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